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Original Article

EFFECTS OF ETHANOL ON MOUSE EMBRYONIC STEM CELL DIFFERENTIATION

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ABSTRACT

Objective: Chronic alcohol consumption during gestation causes fetal malformations, termed fetal alcohol syndrome (FAS). We conducted the present study to clarify the mechanism underlying alcohol consumption-induced malformations.

Methods: First, the effects of ethanol on the viability of cell lines, such as EB3 (undifferentiated mouse embryonic stem (ES) cells), 3T3-Swiss albino, Neuro-2a, NCTC Clone 1469, and UBE6T-15, were determined. Furthermore, ethanol-induced cell death patterns were analyzed by the annexin V-Cy3.18 (AnnCy3) immune fluorescent method. Second, the effects of ethanol on ES cell differentiation were assessed by the embryoid body (EB) model. The formation of an EB, accompanied by spontaneous pulsation derived from EB3 cells, was monitored. EB3 cells were cultured in hanging drops of media containing 0-5% ethanol for 8 days. We then analyzed the EB formation grade by counting the EBs accompanied by spontaneous pulsation in four categories and by monitoring the expression of differentiation marker genes: *connexin43*, *GATA4*, *c-kit*, *α-SMA*, and *Oct-3/4*.

Results: EB3 cells were more sensitive to alcohol than the other four cell lines, and that ethanol-induced death of EB3 cells matched the apoptosis pattern. There were no obvious differences in the formation rates of EBs with pulsation among all ethanol-treated groups. However, *c-kit* gene expression was significantly decreased in the EBs treated with 3 and 5% ethanol, in comparison to the control EBs.

Conclusion: Collectively, the present study suggested that ES cells are more sensitive to ethanol than differentiated cells, and that ethanol-induced down regulation of *c-kit* expression might be involved in alcohol-induced malformations.

Keywords: Fetal alcohol syndrome, Embryonic stem cells, Embryoid body, c-Kit, Apoptosis.

INTRODUCTION

Although ingested alcohol does not bind to either tissue or plasma proteins, it can cross the blood brain barrier and placenta [1]. When alcohol reaches fetal tissue via the placenta, it acts as a teratogenic agent. A higher teratogenic response leads to the fetal malformations termed fetal alcohol syndrome (FAS), which are characterized by microcephaly, neurologic abnormalities, combine dys morphology, and pre-and post-natal growth retardation [2]. Various studies have probed the underlying mechanisms of this alcohol-induced teratogenic action. One possible mechanism is alcohol-induced alimentary tract damage. Rat pups exposed to ethanol *in utero* displayed delayed postnatal development of the intestinal epithelium, leading to impaired nutrient assimilation and growth retardation during postnatal development [3]. It is likely that impaired nutrient absorption may lead to a wide range of disorders. The induction of reactive oxygen species (ROS) by alcohol is also a crucial event for fetal organ development. Maternal ethanol consumption during pregnancy enhanced bile acid-induced ROS in fetal rat liver [4]. Ethanol-induced fetal dys morphogenesis in the mouse is diminished by the high antioxidative capacity of mice transgenic for superoxide dismutase [5]. Furthermore, molecular studies have revealed that *Ercc6l*, an SNF2 family gene, may play a role in the teratogenic action of alcohol [6]. Alcohol exposure decreased *Ercc6l* expression in embryonic brain and heart, which are the most commonly, affected organs of FAS. Although many studies have attempted to clarify the mechanisms as described above, the details are still unclear.

About 50 years ago, unusually large extracellular crystals and particles were found to be produced by embryonic chick cells in special tissue culture environments [7]. Their chemical identities could not be determined by conventional cytochemical techniques in those days. Subsequently, numerous studies concerning the cystic structure named the embryoid body (EB), a product of the spontaneous differentiation of embryonic stem (ES) cells [8], has been performed. The EB facilitates the interaction of cells from the ectodermal, mesodermal, and endodermal lineages, recapitulating the developmental kinetics of normal mouse embryonic development [9]. Since the EB is initially formed by inducing ES cell differentiation, this system should be a useful model for the assessment of the development of various tissues, such as vascular structure [10], and the process of ES cell differentiation. There are three major methods to form an EB: suspension culture in culture dishes, culture in methylcellulose semisolid media, and culture in hanging drops [10].

Recently, the number of female alcohol drinkers has been increasing, and thus the risk of FAS may also be increasing in many countries [11, 12]. Therefore, detailed mechanisms of FAS induction must be understood, for the prevention of this disease. In the present study, we sought to obtain clues for understanding the molecular mechanisms underlying alcohol consumption-induced fetal malformations, by using the EB model.

MATERIALS AND METHODS

Culture of mouse ES cells

EB3 cells, a mouse undifferentiated ES cell line, were kindly provided by Dr. Hitoshi Niwa (Center for Developmental Biology, RIKEN, Kobe, Japan). EB3 cells have one inactivated allele of *Pou5f1* and can be cultured without feeder cells [13]. To maintain the undifferentiated state of the EB3 cells, they were cultured on gelatin-coated dishes without feeder cells in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY), 0.1 mM 2-

mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (GIBCO-BRL), 1 mM sodium pyruvate (Sigma), and 1,000 U/ml leukemia inhibitory factor (LIF, GIBCO-BRL).

Culture of cell lines and measurement of cell viability

3T3-Swiss albino cells (mouse embryo fibroblasts), Neuro-2a cells (mouse neuro blastoma cells), NCTC Clone1469 cells (a mouse normal liver-derived cell line), and UBE6T-15 cells (human bone marrow-derived mesenchymal stem cells) were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). All of these cell lines were cultured in DMEM. The cell lines including EB3 cells were seeded at 2.5 X 103 cells/ml. After 48 h of culture, they were exposed to 0, 1, 2, 3, 4, or 5% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After the treatment with ethanol for 96 h, the cells were harvested and the cell viability was measured by a trypan-blue exclusion test. During the 96 h-culture, no media/ethanol was changed. For the maintenance of pluripotency, we previously confirmed that the pluripotency of EB3 cells was maintained for at least 4 days without LIF [14]. In that study, the *Oct 3/4* mRNA was expressed in EB3 cells for 4 days without LIF. Thus, we did not exchange the medium for 96 h in the present study. Even if the effectiveness of LIF became weakened during the experiment, the pluripotency of EB3 cells was certainly maintained.

Analysis of cell death pattern

EB3 cells were seeded at 5 X 104 cells/ml, allowed to adhere overnight, and exposed to 5% ethanol. After a treatment with/without 5% ethanol for 6h, the cells were harvested. To determine the death pattern of the collected cells, the annexin V-Cy3.18 (AnnCy3) immune fluorescent method was performed. To label the cells with AnnCy3, an AnnCy3 apoptosis detection kit (Sigma) was used. The cells were examined by fluorescence microscopy (BX50, OLYMPUS, Tokyo, Japan). The images facilitated the differentiation between the early apoptotic cells (AnnCy3 positive+6-CFDA positive), the necrotic cells (unstained or AnnCy3 positive+6-CFDA negative), and the viable cells (AnnCy3 negative+6-CFDA positive). These methods were performed according to the manufacturers' instructions.

Formation of embryoid bodies (EBs) treated with/without ethanol

We employed the hanging drop method to form EBs. EB3 cells were suspended in DMEM (LIF-free) containing 0, 1, 3, 5 or 7% ethanol, at cell density of 3.3 X 10^4 cells/ml. A fifteen µL drop of the cell suspension medium was attached to the inside of a culture dish cover (hanging drop method). After 8 days of culture, the EBs formed in the hanging drop were observed by microscopy (ECLIPSE TS100, Nikon, Tokyo, Japan). The pulsation, which is an unidentified motion similar to cardiac beating, of the EB was also assessed by microscopy. Twenty EBs were randomly selected and categorized according to our criteria (table 1).

Table 1: Criteria for assessment of EB pulsation

Score	% of surface with pulsation
+++	90%<
++	50%<~<90%
+	<50%
-	No pulsation

Histological analysis

EBs were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with Hematoxylin-Eosin (HE), observed by light microscopy (BX50, OLYMPUS), and recorded by a CCD camera (DP50, OLYMPUS).

Conventional RT-PCR

EB formation was conducted as described in *Formation of embryoid body (EB) treated with/without ethanol*. At the 4th, 6th, and 8th day of cell culture in the hanging drop, the cells were collected. The mRNA was isolated using an mRNA isolation kit (GE Healthcare, Buckinghamshire, UK), and the first strand of cDNA was synthesized from the isolated mRNA primed with random hexamers using M-MLV reverse transcriptase (GIBCO-BRL). To examine the expression of cell differentiation associated genes, we performed PCR with reverse transcription (RT-PCR) assays specific for mouse *connexin43*, *GATA4*, *c-kit*, *α-SMA*, and *Oct-3/4* mRNA. *GAPDH* mRNA was used as an internal standard. The sequences of the primers used in this experiment are listed in table 2. The RT-PCR products were separated on a 2.0% agarose gel and were visualized with ethidium bromide staining. The density of each band was determined by using Scion Image (Scion Corporation, Frederick, MD).

Table 2: Primers for RT-PCR

Gene (mouse)	Sequence	Product size (bps)
Oct-3/4	5'-AGCTGCTGAAGCAGAAGAGG-3'(F)	468
	5'-CCTGGGAAAGGTGTCCTGTA-3'(R)	
Connexin 43	5'-GATGAGAAAGGAAGAGAAGC-3'(F)	588
	5'-TTGTTTCTGTCACCAGTGAC-3'(R)	
α-SMA	5'-GGAAGACAGCACAGCTCTGG-3'(F)	425
	5'-CATAGAGGGACAGCACAGCC-3'(R)	
c-kit	5'-GCATCACCATCAAAACGTG-3'(F)	332
	5'-GATAGTCAGCGTCTCCTGGC-3'(R)	
GATA4	5'-GCCTGTATGTAATGCCTGCG-3'(F)	500
	5'-CCGAGCAGGAATTTGAAGAGG-3'(R)	
GAPDH	5'-AACGGGAAGCTCACTGGCATG-3'(F)	305
	5'-TCCACCACCCTGTTGCTGTAG-3'(R)	

F: forward primer, R: reverse primer

Statistical analysis

The values in the graphs represent the means±SD. The statistical significance was calculated using the unpaired Student's *t*-test and the one-way ANOVA test followed by the Tukey test. Probability values less than 0.05 were considered to indicate significant differences.

RESULTS

Cell viability

We analyzed and compared the viabilities of five cell lines treated with 0, 1, 2, 3, 4, or 5% ethanol and found that EB3 cells were more sensitive to ethanol than the other four cell lines (fig. 1). For example, only 6.53% of EB3 cells survived when they were treated with 4% ethanol for 96 h, while 90.1% of 3T3-Swiss albino, 91.1% of Neuro-2a, 69.0% of NCTC Clone1469, and 69.2% of UBE6T-15cells survived.

Apoptosis

The AnnCy3 immunofluorescent method was performed to analyze the type of cell death. Almost all of the control EB3 cells were stained with 6-CFDA (green) alone. On the other hand, some of the EB3 cells treated with 5% ethanol for 6 h were stained with both AnnCy3 (red) and 6-CFDA (green), which appeared as yellow

fluorescence (fig. 2). These results suggested that the 5% ethanol–treated EB3cells were in the early stage of apoptosis.

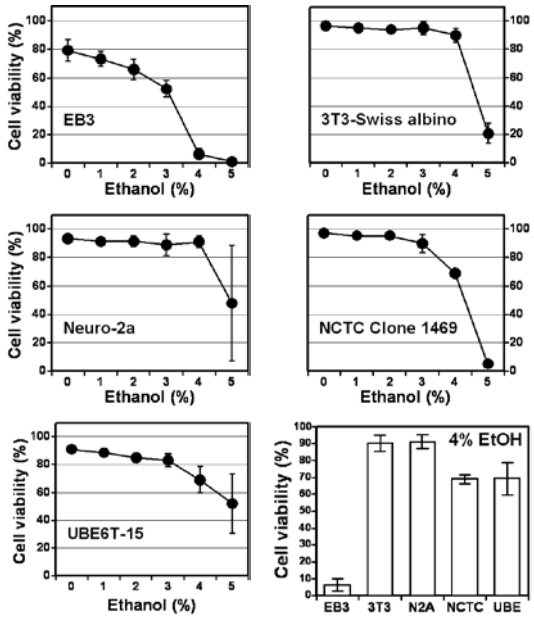

Fig. 1: The viabilities of four mouse cell lines (EB3 cells, 3T3-Swiss albino, Neuro-2a, NCTC Clone 1469) and one human cell line (UBE6T-15) treated with 0, 1, 2, 3, 4, or 5% ethanol were analyzed by a trypan-blue exclusion test. The cell viability data for the 4% ethanol-treated cell lines are also shown as a graph at the bottom of the right column, for comparison between all five cell lines. The data are represented as mean±SD for 5 different experiments. 3T3, 3T3-Swiss albino; N2A, Neuro-2a; NCTC, NCTC Clone 1469; UBE, UBE6T-15; EtOH, Ethanol

Embryoid body (EB) formation

The effects of ethanol on EB formation were observed by microscopy. The cells formed EBs in the presence of ~ 5% ethanol, but not 7% ethanol (fig. 3A). Histological analyses did not reveal obvious differences between the control EBs and 5% ethanol-treated EBs (fig. 3B). In terms of the functions of the EBs, ethanol treatment reduced their pulsation, but there were no obvious differences among the groups treated with different ethanol concentrations (1, 3, or 5%) (fig. 3C). In this study, we used 3.3 X 10^4 cells/ml in the drop (15 µL), and thus 495 cells were considered to be present in the hanging drop. This cell number is reportedly optimal for cardiac differentiation [15]. In this context, the EBs we constructed were similar to a cardiac organ.

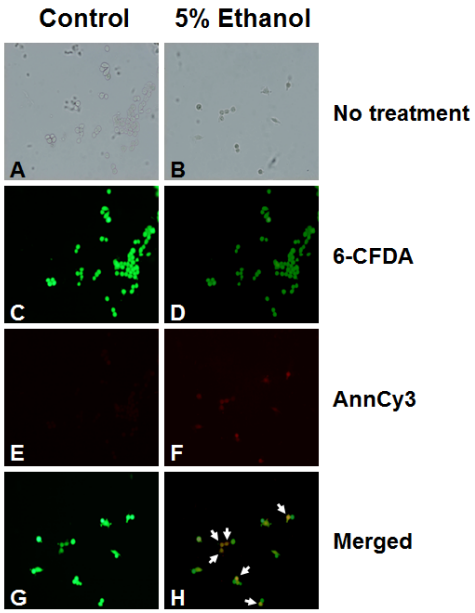

Fig. 2: Cells treated with 5% ethanol for 6 h were examined by the AnnCy3 immunofluorescent method. When cells are incubated with both AnnCy3 and 6-CFDA, living cells and necrotic cells are labeled with 6-CFDA (green) and AnnCy3 (red), respectively. Cells in the early stage of apoptosis will be labeled with both AnnCy3 (red) and 6-CFDA (green). Staining with both AnnCy3 (red) and 6-CFDA (green), which appeared as yellow fluorescence, was frequently observed in cells treated with 5% ethanol. Control EB3 cells showed only living cells period

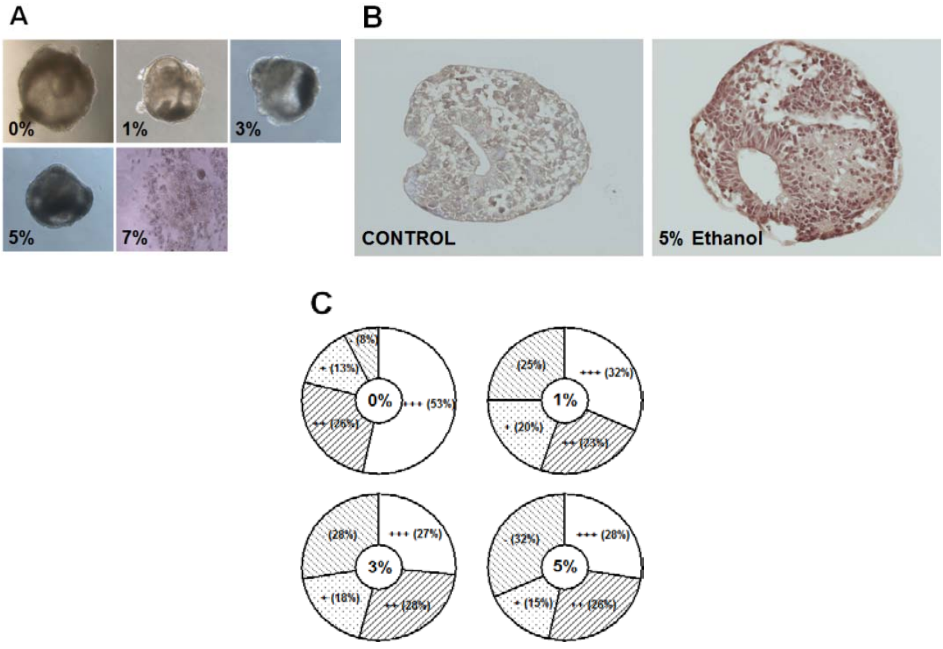

Fig. 3: (A) Photographs of EBs treated with/without ethanol (1, 3, 5, or 7%) are shown. (B) A histological analysis of the EBs is presented. Sections of EBs were stained with Hematoxylin-Eosin (HE). (C) Assessment for the pulsation of EBs treated with/without ethanol (1, 3, or 5%), indicated as a pie chart. Twenty EBs were randomly selected and assessed according to our criteria provided in table 1 period

Expression of cell differentiation associated genes

We analyzed the expression of cell differentiation associated genes, such as *connexin43*, *GATA4*, *c-kit*, *α-SMA*, and *Oct-3/4*, in the EBs treated with ethanol (0, 1, 3, or 5%). Only the expression of *c-kit* was decreased by 3 or 5% ethanol treatment for 8 days, with significant differences in comparison to the control level (fig. 4). The expression of the other four genes showed no significant differences among the ethanol concentration used in this study.

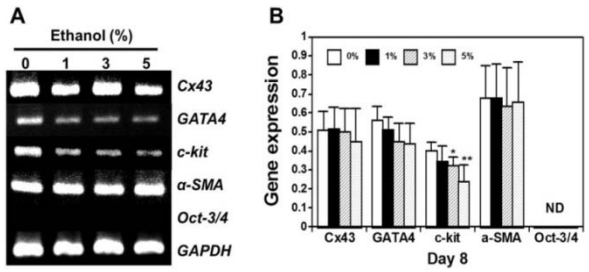

Fig. 4: (A) Expression of cell differentiation-associated genes (*connexin43*, *GATA4*, *c-kit*, *α-SMA*, and *Oct-3/4*) in EBs treated with/without ethanol (1, 3, or 5%), analyzed by conventional RT-PCR. *GAPDH* mRNA was used as an internal standard. (B) The mRNA expression levels of differentiation-associated genes are represented as the ratio to *GAPDH* mRNA. The data are represented as mean±SD for 5 different experiments. Cx43, connexin43; ND, not determined, *P<0.005, **P<0.05 vs. 0% ethanol-treated EB3 cells

DISCUSSION

FAS is caused by ethanol consumption throughout pregnancy, especially during the first trimester. Various mechanisms of ethanol toxicity in fetal development have been presented, although the details are still unclear. We conducted the present study to assess the sensitivity of undifferentiated cells to ethanol, which is possibly related to alcohol consumption-induced fetal malformations, by using the EB model.

The features of ES cells are notable for not only understanding the mechanisms of FAS but also for medical purposes, such as regenerative medicine [16]. Therefore, studies using ES cells would provide a wealth of valuable information. In this study, we first found that ES cells were more sensitive to ethanol exposure than some differentiated cells including human mesenchymal stem cells (fig. 1). Interestingly, almost all of the EB3 cells died by apoptosis upon 5% ethanol exposure (fig. 2), although the EBs that formed exhibited almost normal pulsation even upon 5% ethanol exposure as shown in fig. 3. These results suggested that undifferentiated cells might be more sensitive to ethanol than their differentiated counterparts. The ethanol concentrations we employed in this study (1-7%) were higher than those achieved under physiological conditions or used in other similar research, such as in the report by Arzumanyan *et al.* [17]. However, the issue that we wanted to address in this research was a comparison between ES cells and other differentiated cells, and we found that ES cells were more sensitive to ethanol exposure than other types of cells at the ethanol concentrations of 1-7% we tested.

Since ethanol is known to induce ROS [18], it seemed that the sensitivity of ES cells to ethanol exposure is at least partly due to ROS induced by ethanol. Several studies have suggested that alcohol-induced ROS may be responsible for fetal malformation [4, 5]. However, we previously demonstrated that ES cells were relatively more resistant to ROS than their differentiated counterparts [14]. In addition, recent studies have indicated that the activities of effective antioxidant defense mechanisms in ES cells diminish during differentiation. The defense capacity of ES cells against ROS relies upon the expression of high levels of antioxidant enzymes, such as mitochondrial and cytoplasmic superoxide dismutases, catalase, and peroxiredoxins [19-21], and thus mitochondrial superoxide production and cellular levels of ROS increase during differentiation [21]. Yin *et al.* reported that the amount of reduced GSH decreased in response to the differentiation of ES cells into vascular smooth muscle cells [22]. These studies suggested that the cell defense capacity against ROS is higher in stem cells than in differentiated cells, and becomes attenuated during cell differentiation. In this scenario, the higher sensitivity of undifferentiated cells to ethanol is not attributed to the defense capacity against ROS, suggesting that other mechanisms than ROS production is responsible for the sensitivity of ES cells to ethanol.

C-Kit is a type Ⅲ tyrosine kinase receptor, and its ligand is stem cell factor (SCF). It plays a key role in the maintenance and survival of stem cells, such as hematopoietic stem cells [23], and in cell differentiation, such as melanogenesis, erythropoiesis and spermatogenesis [24]. Several lines of evidence have suggested that c-kit impairment leads to disturbances in tissue development. Cimini *et al.* demonstrated that c-kit dysfunction impairs myocardial healing after infarction [25]. In the present study, we observed that ethanol reduced *c-kit* expression in EBs (fig. 4). Based on these various sources of evidence, including ours, ethanol might disturb organ development partly via the reduction of *c-kit* expression.

CONCLUSION

The higher sensitivity of ES cells than differentiated cells to ethanol exposure may be responsible for alcohol-derived malformations. As a possible mechanism of the higher sensitivity to ethanol, the ethanol-induced down regulation of *c-kit* expression might be involved, leading to organ malformations, such as those in FAS.

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ABBREVIATION

ES cells, embryonic stem cells; EB-embryoid body; LIF, Leukemia inhibitory factor; GATA4, GATA binding protein 4; α-SMA, α-smooth muscle actin.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest period.

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