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Involvement of seven in absentia homolog-1 in ethanol-induced apoptosis in neural crest cells

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ABSTRACT

Ethanol-induced apoptosis in selected cell populations is a major component of pathogenesis underlying ethanol-induced teratogenesis. However, there is a fundamental gap in understanding how ethanol leads to apoptosis in embryos. In this study, we investigate the role of seven in absentia homolog-1 (Siah1) protein, an E3 ubiquitin ligase, in ethanol-induced apoptosis. Using an *in vitro* model of neural crest cell (NCC), JoMa1.3 cells, we found that exposure to 100 mM ethanol resulted in a significant increase in Siah1 mRNA expression in NCCs, an ethanol-sensitive cell population implicated in Fetal Alcohol Spectrum Disorders (FASD). Treatment with 100 mM ethanol for 24 h also significantly increased the protein expression of Siah1 in JoMa1.3 cells. The nuclear translocation and accumulation of Siah1 was evidenced in the cells exposed to ethanol. In addition, we have found that the inhibition of Siah1 function with siRNA prevents ethanol-induced increase in Siah1 protein expression and nuclear translocation in NCCs. Down-regulation of Siah1 by siRNA also greatly diminished ethanol-induced cell death and caspase-3 activation, indicating that inhibition of Siah1 can attenuate ethanol-induced apoptosis. These results strongly suggest that Siah1 plays an important role in ethanol-induced apoptosis in NCCs.

1. Introduction

Fetal Alcohol Spectrum Disorders (FASD) is a term which refers to a range of structural and functional birth defects caused by prenatal exposure to alcohol, with full-blown Fetal Alcohol Syndrome (FAS) representing the severe end of the spectrum. The defects caused by prenatal ethanol exposure include physical, behavioral, mental, and learning disabilities. FASD is considered to be the leading preventable causes of mental retardation (Abel and Sokol, 1986; Burd, 2004).

Accumulated evidences support that excessive cell deaths in selected cell populations is one of the major components of the pathogenesis underlying ethanol-induced teratogenesis. Studies have shown that ethanol exposure resulted in excessive cell death in specific regions of the brain of gestational day 8 to 9 mouse embryos (Dunty et al., 2001; Kotch and Sulik, 1992b). These results have been confirmed by the studies in chick (Cartwright and Smith, 1995a, 1995b). In addition, ethanol-induced cell death has been found in postmitotic neurons in the hypothalamus (De et al., 1994), cerebral cortex (Olney, 2003; Young et al., 2003), cerebellum (Goodlett and Johnson, 1997; Tran et al., 2005), and associated brain-stem structures (Napper and West, 1995). Using the TUNEL technique, it has been shown that ethanol-induced cell death in early embryos is apoptotic (Chen et al., 2001, 2004; Dunty et al., 2001). These results have been confirmed by studies that have shown that ethanol-induced cell death in early embryos can be prevented by compounds that inhibit caspase activation (Cartwright et al., 1998).

Among the sensitive cell populations that are vulnerable to ethanol-induced apoptosis is neural crest cells (NCCs) (Cartwright and Smith, 1995b; Chen and Sulik, 1996, 2000; Kotch and Sulik, 1992b; Sulik et al., 1981). The NCC is a multipotent cell population that can give rise to a diversity of neural and nonneural cell types, such as melanocytes, neurons, glial cells, endocrine cells as well as mesenchymal cells (Delfino-Machin et al., 2007; Hall, 2008; Teng and Labosky, 2006). Cranial neural crest cells can differentiate into a wide variety of structures, including the connective tissues, cartilage, and skeletal structures of the head and facial features (Smith, 1997).

A number of studies have demonstrated that NCCs are particularly vulnerable to alcohol-induced apoptosis (Cartwright and Smith, 1995a; Chen and Sulik, 1996; Chen et al., 2013a, 2013b; Kotch and Sulik, 1992b; Sulik et al., 1988). Excessive cell death in mouse embryos

Abbreviations: FASD, Fetal Alcohol Spectrum Disorder; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt); NCC, neural crest cell; PUMA, p53 upregulated modulator of apoptosis; Siah1, seven in absentia homolog-1; XIAP, inhibitor of apoptosis protein.

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in the regions enriched with NCCs indicated that alcohol directly damaged this cell population (Sulik et al., 1988). While the embryos have capacity to repair damage, studies have shown that ethanol-induced apoptosis in NCCs contributes heavily to subsequent abnormalities (Cartwright and Smith, 1995a; Kotch and Sulik, 1992a; Smith, 1997; Sulik et al., 1981). Although there are numbers of mechanisms that have been proposed for ethanol-induced apoptosis in NCCs, the molecular pathways directly involved in ethanol-induced apoptosis in NCCs is not clear. Understanding of the molecular mechanisms underlying ethanol-induced apoptosis in NCCs can provide insight into the pathogenesis of FASD and may suggest approaches for the development of rational FASD prevention strategies.

The Drosophila seven in absentia (Sina) protein and its human homolog seven in absentia homolog (Siah) are members of a highly conserved family of E3 ubiquitin ligases (Carthew and Rubin, 1990). The members of this family contain an N-terminal RING domain that binds E2 proteins (Carthew and Rubin, 1990; Hu and Fearon, 1999; Lorick et al., 1999). The Siah is widely expressed in developing mouse embryos and in adult tissues (Della et al., 1993). Siah ligases regulate the ubiquitination and proteasomal degradation of several proteins that are important in a variety of signaling pathways, including transcriptional regulators (Tiedt et al., 2001; Zhang et al., 1998), membrane receptors (Liani et al., 2004; Winter et al., 2008), a microtubule-associated motor protein (Linares-Cruz et al., 1998), and other proteins (Tan et al., 2014). Studies have suggested that Siah plays important roles in a variety of biological processes including cell proliferation, migration, axon guidance, and development (Gutierrez et al., 2006; Hu and Fearon, 1999; Lee et al., 2008; Leung et al., 2014; Lorick et al., 1999; Xu et al., 2006b). In addition, Siah plays a critical role in the induction of apoptosis and tumor suppression (Nemani et al., 1996).

Although it is clear that Siah1 plays a critical role in several cell death paradigms and tumor suppression, the potential roles of this signaling pathway in ethanol-induced apoptosis and teratogenesis have not been investigated. In this study, we investigated the role of Siah1 in ethanol-induced apoptosis in JoMa1.3 cells, an *in vitro* model of NCCs. We found that treatment with ethanol significantly increased the mRNA and protein expression of Siah1 in JoMa1.3 cells. Ethanol exposure also resulted in the nuclear translocation of Siah1. Inhibition of Siah1 function with siRNA prevented ethanol-induced increase in Siah1 expression and nuclear accumulation. Down-regulation of Siah1 by siRNA also significantly diminished ethanol-induced cell death as well as the cleavage and activity of caspase-3, indicating that inhibition of Siah1 can attenuate ethanol-induced apoptosis. These results demonstrated for the first time that Siah1 plays an important role in ethanol-induced apoptosis in NCCs.

2. Materials and methods

2.1. Cell culture and ethanol treatment

NCCs (JoMa1.3 cells) were cultured as described previously (Chen et al., 2013a). Briefly, cells were grown on cell culture dishes coated with fibronectin and maintained in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) at 37 °C in 5% CO2/95% air. For ethanol treatment, NCCs were treated with 100 mM ethanol for 6, 12 or 24 h as described previously. Stable ethanol levels were maintained by placing the cell culture plates in a plastic desiccator containing 100 mM ethanol in distilled water (Yan et al., 2010).

2.2. Siah1 siRNA transfection

Siah1 siRNA transfection was performed as described previously (Chen et al., 2013b). Briefly, JoMa1.3 cells were transfected with MISSION® Siah1-siRNA or negative control siRNA (Sigma-Aldrich, St Louis, MO) at a final concentration of 40 nM in the presence of N-TER™ Nanoparticle siRNA Transfection System (Sigma-Aldrich, St Louis, MO), following the manufacturer's instructions. The cells were harvested 48 h after transfection for experiments.

2.3. Immunofluorescence analysis

Cells grown on coverslips were treated with or without 100 mM ethanol for 24 h. Following the treatment, cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with PBS containing 0.25% Triton X-100 for 10 min. Then the cells were washed in PBS three times for 5 min each, blocked with 1% BSA in PBST for 60 min and then subjected to immunofluorescence staining with goat polyclonal Siah1 antibody (Santa Cruz, Santa Cruz, CA) (dilution 1:100) in 1% BSA in PBST in a humidified chamber overnight at 4 °C. The cells were then washed with cold PBS three times for 5 min each, and incubated with Alexa Fluor® 594 chicken anti-goat IgG (H + L) secondary antibody (Invitrogen, Carlsbad, CA) (dilution 1:250) in 1% BSA in PBST for 1 h at room temperature in the dark. The cells were washed three times with PBST for 5 min each in the dark and then counterstained with 0.2 μg/ml DAPI (DNA stain) for 1 min. After washing with PBS three times for 5 min each, the cells were examined by fluorescence microscopy (Olympus IX71).

2.4. Subcellular fractionation

Cells from control and ethanol treated groups were collected and the nuclear fractions of NCCs were generated using the TransFactor extraction kit (Clontech, Mountain View, CA) following the manufacturer's protocol. Briefly, cells were rinsed twice with ice-cold PBS before they were scraped in ice-cold PBS. Cells were resuspended in lysis buffer, incubated for 15 min., and centrifuged for 5 min. at 450 ×*g*. The cell pellet was resuspended in lysis buffer, homogenized with 15 strokes of a homogenizer, and centrifuged at 10,000 ×*g* for 20 min. Then the crude nuclear pellet was resuspended in buffer and disrupted with 15 strokes of a homogenizer. The nuclear suspension was shaken gently for 30 min at 4 °C and the supernatant was collected by centrifuging at 20,000 ×*g* for 5 min. This fraction is the nuclear extract. The protein concentrations were determined using Pierce® BCA protein assay. Fraction purity was assessed using nuclear marker histone 2B and cytosolic marker IkB-α (Santa Cruz, Santa Cruz, CA). Siah1 nuclear accumulation was analyzed by determining the Siah1 protein levels in nuclear fractions using Western blot.

2.5. Quantitative real-time PCR

The mRNA expression of Siah1 was analyzed using quantitative real-time PCR. Total RNAs were isolated from control and treated NCCs using the QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA, USA). Quantitative RT-PCR was carried out using the FastStart Universal SYBR Green Master qPCR kit (Roche Diagnostics, Mannheim, Germany) on a Rotor-Gene 6000 Real-Time system (Corbett Life Science, Sydney, Australia). The primer pairs used for this analysis are: Siah1: forward: 5′-ACTCTGCCACACACCGAAAAGG-3′; reverse: 5′-ATGA CGGCATCCAAGGAGCCTT-3′. These primer pairs were designed using Primer3 and synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA). All quantitative RT-PCR assays were performed in triplicate. Relative quantitative analysis was carried out by comparing threshold cycle number for target genes and a reference β-actin mRNA.

2.6. Western blotting

Western blotting was performed as described previously (Chen et al., 2013b). The levels of Siah1 and the cleavage of caspase-3 were analyzed with SIAH1 rabbit polyclonal antibody (Abcam, Cambridge, MA) (Schonhoff et al., 2006) and rabbit monoclonal anti-cleaved caspase-3 antibody (Cell Signaling, Beverly, MA), respectively. Mouse monoclonal