Tissue and Cell 51 (2018) 14–23

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Role of drug transporters and heat shock proteins during ethanol exposure to human neural precursor cells and its lineages

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ARTICLE INFO

Keywords:
Ethanol exposure
ABC transporters
HSPs
Neuronal deficits

ABSTRACT

Introduction: Ethanol exposure to developing brain may alter the growth and differentiation of neurological cells resulting in unfavorable pathologies. Earlier studies have provided very limited mechanistic insights of cellular and molecular mechanisms which do not mimic with human situation due to varying cell types and poses potential challenges for investigation. Therefore, the present study was undertaken to evaluate the role of ABC transporters and heat shock proteins mediated response in human neural precursor cells (NPCs) and its lineages during proliferation and lineage differentiation against ethanol exposure.

Methods: Effect of ethanol exposure was examined for neuronal cell survival and variation in cellular phenotype during neurospheres development and lineage differentiation. Generation of reactive oxygen species, and variation in cell cycle was identified along with transcriptional profiling for pluripotent markers (Nestin, NCAM, Sox-2, and Notch-2), drug transporters (ABCB1 and ABCG2) and stress protein (HSP70) during ethanol exposure.

Results: ABC transporters as well as HSP70 mRNA expression was higher during proliferation as compared to differentiation with chronic ethanol (1 M) exposure ($p < 0.01$). Ethanol exposure resulted in higher variability in size and shape of developing neurospheres and decreased ability to form new neurosphere colonies. Significant changes were observed in dendrite development due to late ethanol exposure ($p < 0.0001$).

Conclusion: The present study demonstrated significant role of ABC transporters and HSP70 proteins in providing defense against ethanol-induced damage in human neurological cells. However, the over-expression of ABC transporter and HSP-70 proteins during such pathological conditions do not provide complete defense and additional strategies are required to repair the damage.

1. Introduction

Ethanol is a well-known suppressant of central nervous system (CNS) which causes loss of brain cells and impairment in the development of fetal brain. Several neuro-anatomical studies have suggested that neurological cells derived from different regions of human fetal brain have diversified effects during ethanol exposure. The major effects have been shown on the altered growth and function of neurological cells leading to unfavorable pathologies such as teratogenic and pleiotropic effects (Michaelis and Michaelis, 1994; Cheema et al., 2000; Mooney and Miller, 2001). However, these studies provide very limited mechanistic insights for cellular and molecular mechanisms of ethanol effect which poses potential challenges for further investigations.

The co-existence of multiple mechanisms which may act simultaneously or constitutively differ among various cell types (Michaelis, 1990; Guerri, 1998). Nevertheless, no single putative mechanism can account for the structural and functional characteristics of the cell (Phillips et al., 1989; Schenker et al., 1990; West et al., 1994; Abel and Hannigan, 1995). In addition, absence of suitable human model system to investigate such effects at cellular and molecular level poses critical challenges. Application of human fetal SVZ-derived NPCs may be a viable option for better understanding of major cellular and molecular protective mechanisms against ethanol-induced damage. This may also provide significant information for the compounds that are not exclusive substrates for specific proteins.

Therefore, we developed an ex vivo human neural model system

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https://doi.org/10.1016/j.tice.2018.02.001
Received 1 September 2017; Received in revised form 27 January 2018; Accepted 3 February 2018
Available online 07 February 2018
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using fetal sub-ventricular zone (SVZ)-derived homogeneous population of neural precursor cells (NPCs) and its lineages to study the cellular and molecular changes during direct ethanol exposure. Human fetal SVZ represents a renewable source of progenitors having ability to develop into neurospheres and can further differentiate into neural lineage cells (Vishwakarma et al., 2013). Lineage differentiation of human NPCs is a complex mechanism which is characterized by the outgrowth and molecular specialization of axons and dendrites (Poulain and Sobel, 2010; Vishwakarma et al., 2013, 2014a). Although there are few studies which have evaluated the developmental defects in neuronal cells after ethanol exposure (Daniel et al., 2005; Hicks et al., 2010), data related to human fetal SVZ-derived NPCs developmental mechanisms are limited (Sutherland et al., 2013; Dong et al., 2014). However, the precise cellular and molecular basis of ethanol-induced defects in developing neurological cells needs to be identified.

ATP-binding cassette (ABC) transporters also referred as drug transporters are highly conserved trans-membrane proteins which play key role in endogenous and exogenous cell defense mechanisms against toxic substances (Vishwakarma et al., 2014a). These drug transporters also represent strong candidates involved in key events of human NPCs proliferation and differentiation (Bunting, 2002; Vishwakarma et al., 2014a,b). Another important molecule, HSP70 plays crucial role in tissue defense mechanisms due to its chaperon activity. Over expression of these chaperons has been shown to provide defense against stress-induced cell damage (Yang et al., 2008; Vishwakarma et al., 2014b). The interesting fact about ABC transporters and HSP70 is their cumulative protective behaviour in response to an array of chemical/biological stimuli (Vishwakarma et al., 2017). Hence better understanding of ABC transporters and HSP-70 mediated defense in regulation of developmental mechanisms of human neurological cells needs to be elucidated extensively during direct ethanol exposure.

Therefore, the present study was designed to primarily investigate some of the major candidate mechanisms in human fetal neurological cells during ethanol exposure to better understand the molecular regulation of human NPCs fate. The specific objectives of the present study define the ethanol effect on 1) growth factor mediated cell proliferation and survival; 2) neuronal dysfunction due to disturbances in the redox balance; 3) cell cycle progression; 4) neurospheres development; 5) early and delayed response on retinoic acid mediated maturation defects in neuronal cell survival and dendritic development; and 6) changes in quantitative expression of regulatory genes. Studying these mechanistic properties in human neurological cells pre and post ethanol exposure may provide better insight for designing effective therapeutic strategies against ethanol-induced CNS damage.

2. Materials and methods

2.1. Cells isolation and culture

Cells derived from cryopreserved human fetal SVZ (12wk gestation) tissue were used to perform this study. Single cell suspension was prepared and enriched by magnetic activated cell sorting (MACS) using CD133. CD133 + ve cells enriched NPCs were cultured as suspension to observe their proliferation efficiency (Vishwakarma et al., 2013).

2.2. In vitro ethanol exposure to developing neurospheres

To determine the effect of ethanol exposure on neurosphere forming ability, CD133 + ve cells were cultured as described above and exposed to different concentrations of ethanol (10 μM, 100 μM, 10 mM, 100 mM and 1 M) for 14 days. Although 1 M ethanol concentration is well beyond the potential consumption level it was included to complete the dose-response curve. After 14 days the numbers of neurospheres $\geq$100 μM in diameter in each well were counted and compared with the control.

2.3. Cell cycle analysis

Proliferating human NPCs treated with three different concentrations of ethanol

(10 μM, 100 μM and 1 M) for 14 days were fixed in 4% paraformaldehyde (PFA) and stained with propidium iodide (PI) for cell cycle analysis using flow cytometry.

2.4. Neuronal differentiation of ethanol pre-treated NPCs

NPCs derived from primary neurospheres at day 14 were subjected to neural differentiation by using differentiation induction medium (Stem Cell Technologies, Canada). Briefly; 2×10^3 cells were cultured for 14 days at 37 °C with 5% CO_2 and 95% humidity in differential medium supplemented with retinoic acid (RA, 0.5 μM) and 2% fetal bovine serum (FBS) (with and without ethanol). Neurospheres treated with three different concentrations (10 μM, 100 μM and 1 M) of ethanol were used to test differentiation potential of ethanol-treated NPCs. Cells derived from neurospheres without ethanol exposure were used as control.

2.5. Immunofluorescence staining

Existence of neuronal cells in differentiated culture was assayed for the expression of

β tubulin-III using immune-fluorescence staining protocol. Briefly; after 14 days of initial seeding, cover slips with differentiated neuronal cells were fixed in 4% PFA and stained with neuronal cell specific intracellular marker β tubulin-III-FITC and observed under the fluorescence microscope (Carl Zeiss, Germany). Percentage of β tubulin-III positive cells were estimated by flow cytometry analysis and further verified by RT-qPCR.

2.6. Early and delayed ethanol exposure

To examine the effect of ethanol on neuronal cell phenotype, enriched CD133 + ve cells were pre-treated with 100 μM concentration of ethanol in two groups. Early exposure was done at 4 h, 24 h and 48 h of culturing in suspension to generate neurospheres. However delayed exposure was performed at day 7 and 14 after cell seeding. Cells were harvested from both the groups at day 21 and differentiated using RA + 2%FBS induction medium for 21 days.

2.7. Morphological assessment of developing neurons

The morphometric analysis of neurons derived from ethanol pre-treated NPCs were performed by phase contrast microscopy. Neuronal cell morphology and dendritic development was carefully examined in each treatment group. The number of dendrites per cell was counted and compared with control. Size of dendrites per cell was measured in each group.

2.8. Cell survival and growth curve analysis using tetrazolium reduction assay

Cell proliferation and differentiation in ethanol pre-treated cells was assessed by measuring metabolic activity of cells using tetrazolium reduction assay. Briefly; to determine the effect of ethanol on cellular viability, 500 cells/μl cells were seeded in each well of 96 well plates in serum free medium and treated with 10 μM–1 M concentration of ethanol for 21 days. MTT assay was performed at day 1, 7, 14 and 21. Absorbance was measured at each time points at 570 nm by using microplate reader (BIORAD).

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