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Dose effect of gestational ethanol exposure on placentation and fetal growth

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ABSTRACT

Introduction: Prenatal ethanol exposure compromises fetal growth by impairing placentation. Invasive trophoblastic cells, which mediate placentation, express the insulin-IGF regulated gene, aspartyl-asparaginyl β-hydroxylase (ASPH), which has a critical role in cell motility and invasion. The aims of this study were to characterize effects of ethanol on trophoblastic cell motility, and assess ethanol dose-dependent impairments in placentation and fetal development.

Methods: Pregnant Long Evans dams were fed with isocaloric liquid diets containing 0%, 8%, 18% or 37% ethanol (caloric content) from gestation day (GD) 6 to GD18. Fetal development, placental morphology, density of invasive trophoblasts at the mesometrial triangle, as well as placental and mesometrial ASPH and Notch-1 protein expression were evaluated. Directional motility of control and ethanol-exposed HTR-8/SVneo cells was assessed by ATP Luminescence-Based assay.

Results: Severity of fetal growth impairment correlated with increasing doses of ethanol. Ethanol exposure produced dose-dependent alterations in branching morphogenesis at the labyrinthine zone, and inhibited physiological transformation of maternal arteries. ASPH and Notch-1 protein expression levels were reduced, corresponding with impairments in placentation.

Discussion: Prenatal ethanol exposure compromises fetal growth and placentation in a dose-responsive manner. Ethanol's adverse effects on placental development are mediated by: 1) altered branching morphogenesis in labyrinthine zone; 2) suppression of invasive trophoblastic precursor cells; and 3) inhibition of trophoblastic cell adhesion and motility, corresponding with reduced ASPH and Notch-1 protein expression.

1. Introduction

Fetal alcohol syndrome (FAS) is the leading preventable cause of birth defects and developmental disorders in the United States [1]. The severity of alcohol-induced physical defects, and cognitive and behavioral disabilities varies by the timing, dose and duration of the alcohol exposure, nutritional status, genetic polymorphisms, maternal characteristics (gravity, parity, body mass index), and additional exposures (smoking/drugs); collectively, the phenotype is termed "Fetal Alcohol Spectrum Disorders" (FASD) [2–5].

Intrauterine growth restriction (IUGR) is a key feature of FASD. Previously, we showed that prenatal ethanol exposure impairs fetal growth and placentation [6]; placentation is crucial for establishing the maternal–fetal interface needed for nutrient delivery and waste removal. A critical step in establishing the maternal–fetal interface is that invasive trophoblasts must invade maternal spiral arteries, and thereby disrupt the media and replace the endothelial cells [7,8]. This process transforms the normally small muscular arteries into distended, flaccid, high-flow, low-resistance vessels, enabling continuous nutrient supply through placenta to the fetus. Prenatal ethanol exposure disrupts this process [6]. Mechanistically, ethanol inhibits insulin/insulin-like growth factor (IGF) signaling and expression of

Abbreviations: ASPH, aspartyl-asparaginyl β-hydroxylase; GD, gestation day.

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downstream target genes, including aspartyl-asparaginyl β-hydroxylase (ASPH) [6,9].

ASPH is an insulin/IGF responsive gene whose hydroxylase activity regulates cell motility and invasiveness via activation and enhancement of Notch signaling [10–12]. ASPH is a positive regulator of trophoblastic cell motility. Previous in vitro and in vivo experiments demonstrated that siRNA inhibition of ASPH expression inhibits trophoblastic cell motility and alters signaling through Notch-1 leading to decreased expression levels of Notch's downstream target gene Hairy and Enhancer of Split 1 (Hes-1) [13]. The present study was designed to examine ethanol dose-effects on trophoblastic cell motility in relation to ASPH expression and fetal development.

2. Materials and methods

2.1. In vivo model

Pregnant Long Evans rats were fed isocaloric liquid diets (BioServ, Frenchtown, NJ) containing 0%, 8%, 18%, or 37% ethanol by caloric content [14]. The liquid diets were initiated on gestation day (GD) 6 and ended on GD18. After mating, onset of pregnancy (gestation day 0; G0) was confirmed by both the presence of sperm and characteristic metestrous stage cellular changes in the vaginal smears [15]. The dams were weighed on GD 0 and weekly to monitor weight gain. Fetuses, placentas, and maternal serum were harvested on GD 18. Placental weights and fetal body weights and crown-rump lengths were measured. GD was confirmed according to fetal developmental stage [16]. Placental tissue with underlying mesometrial triangle (implantation site) was weighed and either immersion fixed whole in Histochoice, and embedded in paraffin, or divided to separately snap freeze placenta and mesometrial triangle [6]. Adjacent histological sections were stained with hematoxylin and eosin (H&E) to study structural alterations associated with ethanol exposure, and immunohistochemical staining to assess shifts in invasive trophoblast cell populations. Frozen tissue was stored at −80 °C. The dams' blood alcohol levels were measured using a GM7 analyzer (Analox Instruments Ltd., Lunenburg, MA) according to the manufacturer's protocol. The Institutional Animal Welfare Committee at Rhode Island Hospital approved this study.

2.2. Immunohistochemistry

Histological sections of placenta with underlying mesometrial triangle (5 μm thick) were immunostained with monoclonal antibody to cytokeratin (CK) to detect invasive trophoblastic cells at the implantation site [13,17]. Three samples per litter were included in the evaluation (N = 21, 0%; N = 15, 8%; N = 18, 18%; N = 27, 37%). Deparaffinized, rehydrated tissue sections were incubated in preheated EnVision™ FLEX Target Retrieval Solution (high pH = 9.0) for 20 min at 95–100 °C. Slides were then rinsed in EnVision™ FLEX wash buffer and treated with 3% hydrogen peroxide for 5 min to quench the endogenous peroxidase activity. The slides were incubated for 20 min at room temperature with a 1:50 dilution of monoclonal cytokeratin antibody (DAKO #M0821, clone MNF-116). Antibody binding was detected with EnVision™ FLEX/HRP reagent (dextran backbone coupled to a large number of HRP molecules) and diaminobenzidine tetrahydrochloride (DAB) as the chromogen. The sections were counterstained lightly with hematoxylin and preserved under coverglass. All immunohistochemical staining reactions were performed using the Dako Autostainer (Dako, Carpenteria, CA).

2.3. Stereology

We quantified the trophoblastic giant cells in H&E stained sections and invasive trophoblastic cells in CK-stained sections using unbiased stereology [13]. Numerical density calculations were performed with the aid of an Olympus BX60 light microscope (Olympus America Inc., Center Valley, PA) with attached MS-2000 XYZ Inverted Stage (Applied Scientific Instrumentation, Eugene, OR), and Stereologer software (Stereology Resource Center, Inc., Chester, MD). The anatomical area of interest was the entire junctional zone for trophoblastic giant cell quantification, whereas, the entire mesometrial triangle extending up to the placental junctional zone was selected as the anatomical area of interest for quantifying CK-positive invasive trophoblasts. The upper boundary of the mesometrial triangle was delineated by the diffuse CK-immunoreactivity of junctional zone. Sections showing linear maternal spiral arteries with maternal channel continuation were selected for the evaluation to maintain uniformity. Unbiased counting frames were applied under software control. Cells of interest were counted at 400X magnification using optical dissector method and normalized to volume based on the Cavalieri point grid method [13].

2.4. Enzyme-linked immunosorbant assay (ELISA)

Direct binding enzyme-linked immunosorbant assay was used to measure ASPH and Notch-1 immunoreactivity. Control and ethanol-exposed placental and mesometrial triangle tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitors using a TissueLyser II apparatus (Qiagen Inc., Valencia, CA) [18,19]. Protein samples were prepared as described above. Samples containing 50 ng of protein in 100 μl Tris-buffered saline, pH 7.4 (TBS) were adsorbed to the bottom flat surfaces of opaque white Maxisorp polystyrene 96-well plates (Nunc ThermoScientific, Rochester, NY) overnight at 4 °C. Non-specific binding sites were blocked by a 3-h room temperature incubation with 300 μl TBS +0.05% Tween 20 + 3% BSA. Samples were then incubated with either A85G6 monoclonal antibody (mAb) to ASPH or Notch-1 mAb (0.5–1.0 μg/ml) for 1 h at 37 °C. Antibody binding was detected with HRP-conjugated secondary antibody (Pierce, Rockford, IL) and the Amplex UltraRed soluble fluorophore (Molecular Probes, Eugene, OR) [19]. Immunoreactivity was measured (Ex 530/Em 590) in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). The results were normalized to protein content in each well as quantified using the NanoOrange® Protein Quantitation Kit (Molecular Probes, Eugene, OR). Negative control reactions included omission of protein sample, or the primary antibody, secondary antibody, or both antibodies. Between steps, the wells were washed 3 times with TBST using a Nunc Immunowash apparatus (Nunc, Rochester, NY).

2.5. In vitro model

HTR-8/SVneo cells are immortalized, first trimester, human trophoblast cells that have properties of invasive extravillous cytotrophoblasts [20]. Cultures were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Lonza Walkersville, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) and 2.1 mM L-glutamine at 37 °C in a 5% CO_2 incubator. After determining the optimal ethanol dose based on ethanol dose-response studies that utilized cell viability and toxicity assays (data not shown), cells were exposed to 0 mM or 128 mM vaporized ethanol for 48 h in sealed humidified chambers [21] and then harvested for motility assays and Western blot analysis.

2.6. Directional motility assay

Directional motility was measured using the ATP Luminescence-Based Motility-Invasion (ALMI) assay [22]. Briefly, blind-well chambers (Neuro Probe, Gaithersburg, MD) partitioned by polycarbonate filters with 12 μm pore diameter were used for the experiments. Culture medium containing 1% FBS and 0.1 μg/ml IGF2 as the trophic factor was placed in the lower chambers and 100,000 viable (Trypan blue excluded) control and ethanol-exposed cells, suspended in 100 μl serum-free medium, were seeded into the upper chamber. Cell migration was allowed to proceed for 30 min at 37 °C in a CO_2 incubator. Cells harvested from the upper chamber (sessile), undersurface of the membrane (motile, adherent), and lower chamber (motile non-adherent) were quantified using ATPlite reagent, and luminescence was measured in a TopCount machine (Perkin–Elmer, Waltham, MA). The percentages of sessile (non-motile), motile adherent, motile non-adherent, and total motile (adherent + non-adherent) cells were calculated, and statistical analyses were performed using results from 8 replicate assays.

2.7. Western blot analysis

Western blot analysis was used to measure ASPH immunoreactivity. Control and ethanol-exposed HTR8 cells were homogenized in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA) containing protease (1 mM PMSF, 0.1 mM TPCK, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 1 mM NaF, 1 mM $Na_4P_2O_7$) and phosphatase (2 mM Na_3VO_4) inhibitors. Supernatant fractions obtained after centrifuging the samples at 14,000 × g for 10 min at 4 °C were used in Western blot assays as described [23]. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The blots were probed with the A85G6 monoclonal antibody (mAb) to ASPH (0.5–1 μg/ml) [6,23] and immunoreactivity was detected using horseradish peroxidase (HRP)-conjugated secondary antibody and SuperSignal enhanced chemiluminescence reagents (ECL, Pierce Chemical Company, Rockford, IL) as described. Western blot signals were quantified by digital imaging with the Kodak Digital Science Imaging Station (NEN Life Sciences, Boston, MA). Sample loading was assessed by re-probing stripped blots with polyclonal anti-p85 (subunit of PI3 kinase) [10].

2.8. Sources of reagents

Monoclonal antibody to cytokeratin (CK clone MNF-116) was purchased from Dako (Carpenteria, CA). Polyclonal antibody to p85 was purchased from Millipore (Temecula, CA). The 85G6 ASPH monoclonal antibody was generated to human recombinant protein and purified over a Protein G column (Healthcare, Piscataway, NJ) [6,23]. Monoclonal antibody to Notch-1 was purchased from Abcam Inc. (Cambridge, MA). Histochoice fixative was purchased from Amresco Corp. (Solon, OH). BCA reagents were purchased from the Pierce Chemical Company (Rockford, IL). ATPLite substrate was obtained from Perkin–Elmer Corp. (Waltham, MA). Polycarbonate filters used in directional motility assays were purchased from GE Osmonics Labstore (Minnetonka, MN). All other fine chemicals and reagents were purchased from Calbiochem-EMD Biosciences (La Jolla, CA), Pierce Biotechnology Inc. (Rockford, IL), or Sigma–Aldrich (St. Louis, MO).