

BRIEF COMMUNICATION

Novel window on early human neurodevelopment via fetal exosomes in maternal blood

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Funding Information

This work was supported by Bill and Melinda Gates Foundation.

Received: 23 November 2015; Revised: 21 January 2016; Accepted: 27 January 2016

Annals of Clinical and Translational Neurology 2016; 3(5): 381–385

doi: 10.1002/acn3.296

Introduction

The prevalence of autism, attention deficit hyperactivity disorder (ADHD), and other neurodevelopmental conditions with potential in utero etiologies have been increasing over time.¹ In utero exposure to chemicals, medications, infections/excess inflammation, or unfavorable nutritional conditions can disrupt fetal brain development, deplete subpopulations of neurons and adversely affect the formation of normal synaptic connections.^{2,3} A major roadblock to unraveling the mechanisms and timing of neurodevelopmental derangement is the almost complete absence of sensitive noninvasive assessments. Recent advances in prenatal diagnosis demonstrate that fetal DNA can both cross the placenta and be isolated from first trimester maternal blood.^{4–8} In adult populations, we have shown that neural exosomes cross the mature blood brain barrier (BBB) and can be isolated for detection of preclinical Alzheimer's disease.^{9–12} Our recent findings provided the impetus to determine if fetal neural exosomes (FNEs) could cross the immature BBB and placenta, and be isolated from the

Abstract

Adverse in utero exposures can disrupt fetal brain development, deplete subpopulations of neurons and inhibit formation of normal synaptic connections. A major roadblock to unraveling the precise mechanisms and timing of human neurodevelopmental derangement is the almost complete absence of sensitive noninvasive assessments. We present novel methods for isolating fetal neuronal exosomes from maternal plasma as a noninvasive platform for testing aspects of fetal neurodevelopment as early as the 1st trimester. Our methodology represents an important breakthrough both in understanding mechanisms of injury in vivo in a human system and potentially for monitoring clinical interventions seeking to promote fetal brain health.

maternal compartment as a noninvasive platform for testing aspects of fetal neurodevelopment (http://gcgh.grandchallenges.org/Explorations/Topics/Pages/MeasureBrainDevelopment_Round13.aspx). Here we present novel methods for isolating FNEs from maternal plasma in early pregnancy. Further, as a proof of this concept, we present data to suggest that decreased fetal neuronal defenses can be detected following in utero exposure to the known fetal neurotoxin ethanol as early as the 1st trimester. Our methodology represents an important breakthrough, both in understanding the mechanisms of injury in an in vivo human system and in guiding and monitoring interventions seeking to promote fetal brain health.

Methods**Patient groups and blood sampling**

Under an institutional review board approved protocol, plasmas were obtained from healthy pregnant women in the first or second trimester (healthy pregnancy [HP],

$n = 20$), from gestational age matched pregnancies with current heavy EtOH use Fetal Alcohol Syndrome (FAS, $n = 10$) and from nonpregnant controls ($n = 16$). Twenty mL of venous blood were drawn into 1 mL of saline with EDTA or heparin, incubated for 10 min at room temperature and centrifuged for 15 min at 2500g. Plasma was stored in 0.5 mL aliquots at -80°C .

Isolation of FNEs from plasma for extraction and ELISA quantification of exosomal proteins

Two hundred-fifty μL of plasma was incubated with 100 μL of thromboplastin-D (Fisher Scientific, Inc., Hanover Park, IL) and then cocktails of protease and phosphatase inhibitors as described.^{9,10} After centrifugation, supernates were incubated with exosome precipitation solution (EXOQ; System Biosciences, Inc., Mountainview, CA), resultant suspensions centrifuged at 1500g for 30 min at 4°C , and pellets resuspended in 400 μL of distilled water with inhibitor cocktails for immunochemical enrichment of exosomes.^{9,10} To isolate exosomes from fetal neural sources, total exosome suspensions were incubated for 90 min at 20°C with 50 μL of 3% Bovine Serum Albumin (BSA) (Thermo Scientific, Inc., Waltham, MA) containing 2 μg of mouse monoclonal IgG1 antihuman Contactin-2/TAG1 antibody (clone 372913, R&D Systems, Inc., Minneapolis, MN), that had been biotinylated (EZ-Link sulfo-NHS-biotin System, Thermo Scientific, Inc.). Then 10 μL of Streptavidin-Plus UltraLink resin (Pierce-Thermo Scientific, Inc.) in 40 μL of 3% BSA were added and the incubation continued for 60 min at 20°C .^{11,12} After centrifugation at 300g for 10 min at 4°C and removal of supernates, pellets were resuspended in 75 μL of 0.05 mol/L glycine-HCl (pH 3.0), incubated at 4°C for 10 min and recentrifuged at 4000g for 10 min at 4°C . Each supernate in a new 1.7 mL Eppendorf tube was mixed with 5 μL of 1 mol/L Tris-HCl (pH 8.0) and 20 μL of 3% BSA followed by addition of 0.40 mL of M-PER (mammalian protein extraction reagent; Thermo Scientific, Inc.) containing protease and phosphatase inhibitors prior to storage at -80°C . For exosome counts, immunoprecipitated pellets were resuspended in 0.25 mL of 0.05 mol/L glycine-HCl (pH = 3.0) at 4°C , suspension pH adjusted to 7.0 with 1 mol/L Tris-HCl (pH 8.6) and exosome suspensions diluted 1:200 to permit counting in the range of $1-5 \times 10^8/\text{mL}$, with an NS500 nanoparticle tracking system (NanoSight, Amesbury, U.K.), as described.¹⁰

The tetraspanning exosome marker human CD81 (American Research Products, Waltham, MA-Cusabio) and neural markers neuron-specific (NS)-enolase (R&D Systems, Inc.), neurofilament light (NF-L) chain (American Research Products-Cusabio) and L1-cell adhesion

molecule (L1CAM) (Biomatik, Wilmington, DE) were quantified by human-specific ELISAs according to suppliers' directions. Exosomal cargo levels of human Sonic Hedgehog (Abcam, Inc., Cambridge, MA), type 1 pregnancy-specific β -1-glycoprotein (PSG-1) (R&D Systems, Inc.), type 1 heat-shock factor (HSF1) (Enzo Life Sciences, Inc., Farmingdale, NY), B-cell lymphoma-extra large (Bcl-XL) (American Research Products-Cusabio), and restriction element-1 silencing transcription factor (REST) (American Research Products-Cusabio) were also quantified by ELISAs. The mean value for all determinations of CD81 in each assay group was set at 1.00 and the relative values for each sample was used to normalize their recovery.

Preparation of placental tissue to exclude placental origin of maternal serum exosomes

Placental tissue was weighed and homogenized at 1 g/mL to approximate tissue fluid levels of proteins in M-PER with protease and phosphatase inhibitors using a motorized pestle system adapted to 1.5 mL Eppendorf tubes (Bel-Art, Thomas Scientific Co., Swedesboro, NJ) for 3 min at 4°C . Homogenates were centrifuged at 2000g for 10 min at 4°C and supernates were stored at -80°C in 100 μL aliquots.

Results

What proportion of circulating neuronally derived exosomes are fetal in origin?

Contactin-2/TAG1 is a glycosylphosphatidylinositol-anchored neuronal membrane adhesion protein of the immunoglobulin superfamily that is transiently expressed in early human developmental stages to guide initial axonal connections and, in association with other proteins, promote molecular organization of myelinated nerves.^{13,14} A monoclonal antibody specific for contactin-2 immunoabsorbed a subset of plasma exosomes, considered to be FNEs, from physically precipitated and resuspended total plasma exosomes of pregnant women. Counts of immunoabsorption-enriched FNEs were a mean ($n = 3$ in each group) of 1.04×10^8 , 0.86×10^8 and $<10^7$ per mL of plasma, respectively, in HP, FAS pregnancies, and nonpregnant control women. In contrast, counts of total neural exosomes that had been immunoabsorption-enriched with anti-L1CAM antibody, as described,¹¹ were a mean ($n = 3$ in each group) of 5.47×10^8 , 4.96×10^8 and 5.22×10^8 , respectively, in HP, FAS pregnancies, and nonpregnant control women. The preliminary conclusion was that FNEs constitute

~20% of the total set of neural exosomes in the plasma of women in the first/second trimester of pregnancy.

Evidence to support fetal origin of exosomes

The identity of FNEs recovered from plasma was supported by quantification of their protein markers

(Table 1). CD81, a general exosome marker, suggested that FNE levels were similar in HP and FAS pregnancies. In samples prepared from nonpregnant control women CD81 levels were only 3% of pregnancy values. Relative absence of fetally derived neuronal exosomes was not due to a lack of circulating neuronal exosomes in nonpregnant controls: total neural exosomes had mean \pm SEM levels of CD81 that were similar at

Table 1. Fetal neural exosome protein markers.

(A) Neural markers					
Fetal neural exosomes	<i>n</i>	CD81	NS-enolase	NF-light chain	L1CAM
Heavy EtOH exposure	10	1075 \pm 66.3	1942 \pm 372	1087 \pm 108	353 \pm 19.2
Healthy pregnancy	10	984 \pm 77.8	2600 \pm 290	989 \pm 81.9	340 \pm 34.8
Nonpregnant controls	16	33.3 \pm 1.87	<100	<20	<10
(B) Fetal and placental markers					
Fetal neural exosomes	<i>n</i>	CD81	Sonic Hedgehog	PSG-1 (ng/mL)	
Heavy EtOH Exposure	10	1075 \pm 66.3	409 \pm 59.6	<3	
Healthy pregnancy	20	784 \pm 63.1	646 \pm 104	5.35 \pm 1.14	
Nonpregnant controls	16	33.3 \pm 1.87	63.3 \pm 14.6	<3	
Placental extracts	6	27.4 \pm 7.47	–	314 \pm 55.6	

Each value is mean pg/mL \pm SEM, except for PSG-1, that is in ng/mL. Differences in CD81 and Sonic Hedgehog between pregnancies with heavy ethanol (EtOH) exposure or healthy pregnancies and nonpregnant controls are significant at $P < 0.0001$, as determined by an unpaired *t* test. Differences in PSG-1 between FAS or healthy pregnancies or nonpregnant controls and placental extracts are significant at $P < 0.0001$, as determined by an unpaired *t* test. NS, neuron-specific; NF, neurofilament; L1CAM, L1-cell adhesion molecule; PSG-1, type 1 pregnancy-specific β -1-glycoprotein.

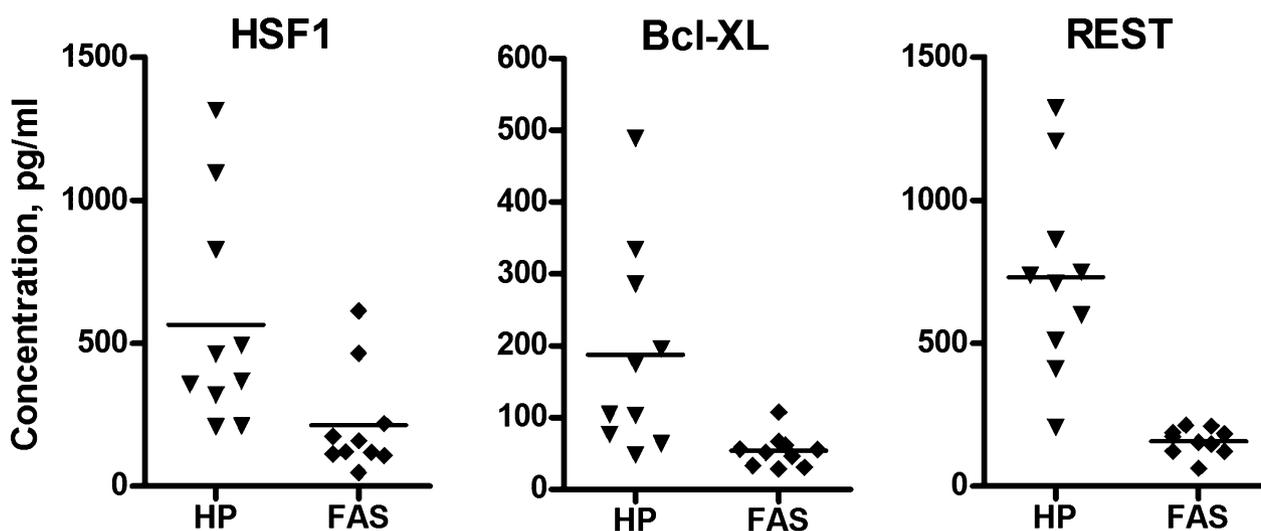


Figure 1. Decreased fetal neural exosome levels of neuronal survival factors in FAS. Each point represents a value for a control HP ($n = 10$) or pregnancy with heavy alcohol exposure (FAS, $n = 10$) and each horizontal line depicts the mean for that group of values. The significance of differences between levels of HSF1 ($P = 0.0267$), Bcl-XL ($P = 0.0092$), and REST ($P < 0.0001$) for the HP and FAS pregnancy groups was calculated by an unpaired *t* test. HP, healthy pregnancy; HSF1, type 1 heat-shock factor; Bcl-XL, B-cell lymphoma-extra large; REST, restriction element-1 silencing transcription factor.

3845 ± 81.7 and 4257 ± 135 pg/mL. Similarly, the levels of neural markers NS-enolase, NF-light chains, and L1CAM were similar in plasma preparations from HP and FAS pregnancies, but much lower in nonpregnant control women (Table 1A). Levels of neural markers in fetal preparations of neural exosomes were indistinguishable from those in total neural exosomes from plasmas of nonpregnant control women (fourfold higher levels of exosomes). This suggests that neural markers appear to be more highly expressed in plasma FNEs than in adult/maternal neural exosomes. Similarly, high levels of the fetal marker Sonic Hedgehog were recovered from plasma FNEs in HP and FAS pregnancies, as contrasted with much lower levels in nonpregnant control women (Table 1B).

Evidence to exclude placental origin of exosomes

The placental marker PSG-1 was readily quantified in placental tissue extracts, but levels in plasma FNEs were undetectable in FAS pregnancies and nonpregnant control women, and only 1.5% of placental tissue levels in plasma FNEs from HP essentially excluding placental origin of the purified exosomes.

Purified FNEs: an effective, noninvasive method of assessing early human neurodevelopment

To assess the resistance of developing fetal neurons to injuries inflicted in the FAS, levels of HSF1 that recruits heat-shock neuronal survival factors,^{15,16} Bcl-XL mitochondrial membrane protein that suppresses mitochondrial-mediated caspase activation, and consequent neuronal apoptosis¹⁷, and REST^{18,19} that maintains brain levels of diverse neuronal defense factors were quantified in plasma FNEs (Fig. 1). Levels of all three neuronal defense proteins were significantly lower in FAS than in HP. REST showed the greatest differences with overlap of only one data point (Fig. 1).

Conclusions

Our results suggest that FNEs purified from maternal plasma may be a powerful tool to assess aspects of fetal neurodevelopment as early as the first trimester. We purposefully targeted the earliest feasible time point in gestation when the minimal mass of the fetal brain would be expected to release the smallest absolute amount of exosomes into the maternal circulation. It is likely that the relative proportion of FNEs, the absolute amount of FNEs, and the subtypes of neuronal cells

represented change across gestation. While FNEs were essentially undetectable in nonpregnant women, we cannot exclude the possibility that FNEs persist in the maternal circulation as do fetal cells.²⁰ However, even if a few FNEs continue into subsequent pregnancies, the expected relatively low level of contamination is unlikely to be problematic. Finally, we present data to suggest that fetal neuronal damage can be detected noninvasively following in utero exposure to EtOH in the 1st trimester. Neuronal injury may be partially attributable to decreased resistance to diverse toxic factors as reflected in lower than normal levels of neuronal survival proteins.^{21–24} Our rationale for selecting these three injury response proteins was our expectation that they would be altered independent of the specific mechanism of neuronal injury and serve as a general marker of 1st and 2nd trimester injury to the developing Central Nervous System (CNS). Our methodology represents an important breakthrough, both in understanding mechanisms of injury in an in vivo human system and supporting findings from animal models where the validity of extrapolation to human development has not always been clear.²⁵ Future investigations should delineate precisely those cellular processes represented within FNE exosomes; the changing composition of FNEs across gestational age; and what aspects of disrupted neurodevelopment are detectable. Our work opens up new opportunities to correlate postnatal neurodevelopmental outcomes with serial noninvasive measures of in utero brain development across gestation. Ultimately, FNE-based assessments may be used to guide and monitor interventions seeking to promote fetal brain health and reduce rising rates of autism, ADHD, and other neurodevelopmental disorders with potential in utero contributions.

Acknowledgments

We acknowledge the Gates Foundation for their generous funding as well as all the patients who participated in this study.

Author Contributions

L. G.: Conception and design of the study; acquisition and analysis of data; drafting the manuscript or figures. N. D.: Design of the study; review of the manuscript or figures. E. J. G.: Conception and design of the study; acquisition and analysis of data; drafting the manuscript or figures.

Conflict of Interest

L. G. and E. J. G. have a patent application pending for some of the methods described in the manuscript.

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