Gene-expression analysis after alcohol exposure in the developing mouse

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Exposure to alcohol in the embryonic mouse can lead to structural and neurophysiologic changes. The cause of these changes is poorly understood, but they are likely the result of numerous mechanisms. Here we investigate ethanol-induced alterations in gene expression in the fetal brain. Using complementary-DNA microarrays, we identified 25 genes that were down-regulated by prenatal ethanol exposure on days 7 and 9 of gestation. None were found to be up-regulated. Of those that were repressed, 6 (*Timp4*, *Bmp15*, *Rnf25*, *Akt1*, *Tulp4*, *Dexras1*) have been identified, and they are discussed here in the context of the developing fetus. The identified genes have been shown to be involved in cell proliferation, differentiation, and apoptosis, and they contribute to tissue growth and remodeling, as well as neuronal growth and survival. Microarray studies may be useful in the identification of a genetic marker for fetal alcohol syndrome, the discovery of novel pathways that may be involved in its origin, or both. (J Lab Clin Med 2005;145:47–54)

Abbreviations: *Akt1* = thymoma viral protooncogene-1; BLAST = Basic Local Alignment Research Tool, a public service offered by the National Centre for Biotechnology Information; *Bmp15* = bone morphogenic protein-15; Cy3 = cyanine-3; Cy5 = cyanine-5; *Dexras1* = dexamethasone-induced RAS-1; cDNA = complementary DNA; dCTP = deoxycytidine triphosphate; dNTP = deoxyribonucleoside triphosphate; ECM = extracellular matrix; EDTA = ethylenediaminetetraacetate; EST = expressed sequence tag; FAS = fetal alcohol syndrome; FASD = fetal alcohol spectrum disorder; FDR = false-discovery rate; GD = gestational day; Gdf9 = growth-differentiation factor-9; GDP = guanine diphosphate; GTP = guanine triphosphate; MAP = mitogen-activated protein; MMP = matrix metalloproteinase; PCR = polymerase chain reaction; *Rnf25* = ring-finger protein-25; SAM = significance analysis of microarrays; SSC = standard saline citrate; TGF-β = transforming growth factor-β; TIMP = tissue-derived inhibitor of metalloproteinases; *Timp4* = tissue inhibitor of metalloproteinase-4; *Tulp* = tubby-like protein; *TUSP* = tubby-superfamily protein; tRNA = transfer RNA

Alcohol consumption during pregnancy often results in abnormal fetal development, both in human beings[1,2] and experimental animal models,[3–7] leading to FASD. The anomalies associated with full-blown FAS include pre- and postnatal growth retardation, distinct craniofacial dysmorphology, and deficits of the central nervous system.[2] Ethanol, which produces its most severe effects on the developing brain, causes a broad array of neuropathologic changes, including microcephaly, reduced volume of cerebral white matter and cerebellar hypoplasia.[8,9] Despite efforts to better understand the origin and molecular

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pathways involved, the mechanisms behind the teratogenic properties of alcohol are still poorly understood.

The deleterious effects of ethanol on the developing mouse are well documented,[10] and several studies have shown that both maternal and fetal genetic factors may influence a fetus' susceptibility or resistance to alcohol exposure.[11–13] In this study we characterized FAS in a mouse model by measuring changes in gene expression in the fetal brain with the use of cDNA microarrays. The results of such studies may not only reveal the mechanisms and potential targets of alcohol toxicity but could also provide an objective biologic marker of FASD.

METHODS

Animals. We chose to study the C57BL/6J inbred mouse strain, which has been shown to respond to ethanol teratogenicity with both structural and neurophysiologic changes.[5,14] Male and female C57BL/6J mice obtained from the Jackson Laboratory (Bar Harbor, Maine) were housed and cared for in a fashion approved by the Animal Care Committee of The Hospital for Sick Children. A 12-hour light cycle (6 AM–6 PM) was maintained, and food and water were provided ad libitum. Male and female mice were mated at 10 weeks of age during the dark cycle. Females were examined for a vaginal plug the next morning; the presence of a plug indicated GD 0. Pregnant mice were weighed on GDs 7 and 9 at 10 AM and treated with a single intraperitoneal dose of 25% (vol/vol) ethanol (2.9 g/kg; $n = 3$) or an equal volume of isotonic saline solution ($n = 3$) on both days. This dosage produces a peak blood-alcohol concentration of approximately 400 mg/dL, sufficient to produce fetal malformation.[14] On GD 18, female mice were killed by means of CO_2 inhalation between 9 and 11 AM. The uterine horns were exposed and fetuses were removed, counted, and weighed. Fetal brains were removed, submerged in liquid nitrogen, and stored at −70°C until RNA isolation was performed.

RNA preparation. We extracted total RNA from homogenate of the total fetal brain with the use of TRIzol reagent (Life Technologies, Burlington, Ontario, Canada) and subsequently cleaned it using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) in accordance with the RNeasy Mini protocol for RNA cleanup. The purity and quantity of the RNA were determined with the use of spectrophotometry. We assessed the integrity of the RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Ontario, Canada). Total RNA extracted from each litter was pooled separately for each of the ethanol-treated groups, yielding 3 separate pools. A single reference pool of RNA was produced from the 3 saline solution–treated groups. A common reference design[15] was used for hybridization to cDNA-spotted 15k mouse arrays (University Health Network Microarray Centre, Toronto, Ontario, Canada); the ethanol-treated samples were labeled with Cy3, the saline solution–treated sample with Cy5. One additional microarray was performed (array 71779) in which the channels were flipped (the samples as for array 71850 were used). All 4 arrays were used in the analysis.

Labeling and hybridization. The probes were prepared in accordance with a modified version of the direct-labeling protocol provided by the Microarray Centre (www.microarrays.ca/support/proto.html). In brief, 10 μg of pooled total RNA was reverse-transcribed with the use of SuperScript II RNase H^- Reverse Transcriptase (Life Technologies) and equal concentrations (2 pmol/μL) of an Anc-T primer (Cortec DNA Service Laboratories, Kingston, Ontario, Canada) and a random hexanucleotide (Cortec DNA Service Laboratories) in the presence of 1.5 mmol/L dNTP-dCTP and 50 μmol/L dCTP (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), and 25 μmol/L of either Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech). The reaction was allowed to proceed for 3 hours at 42°C, with an additional aliquot of reverse transcriptase added to the reaction at 1.5 hours. To stop the reaction, we added 4 mL of 50 mmol/L EDTA and 2 mL of 10N NaOH to hydrolyze the remaining RNA and added 4 μL of 5M acetic acid to neutralize the mixture. The Cy3 (ethanol-treated) sample and Cy5 (saline solution–treated sample) reaction mixtures were combined and the labeled cDNA collected with the use of Millipore Microcon polymerase chain reaction filter units (Fisher Scientific, Nepean, Ontario, Canada). In array 71779, the saline solution–treated sample was labeled with Cy3 and the ethanol sample was labeled with Cy5.

The hybridization solution was a 20:1:1 ratio of DIG Easy Hyb (Roche, Mississaug, Ontario, Canada), sonicated calf-thymus DNA (Sigma-Aldrich, Oakville, Ontario, Canada), and yeast tRNA (Life Technologies), which was incubated at 65°C for 2 minutes and then left at room temperature. The combined Cy3/Cy5 sample was added to 75 μL of the hybridization solution, mixed with the use of gentle up-and-down pipetting and then incubated at 65°C for 3 minutes. The sample was loaded onto the microarray and incubated in a hybridization chamber overnight at 37°C. After hybridization, the microarray was washed 3 times for 10 to 15 minutes in 1× SSC, 0.1% sodium dodecyl sulfate solution at 50°C after removal of the coverslip in room-temperature 1× SSC. The slide was rinsed in room-temperature 1× SSC, then 0.1× SSC. The slide was dried at 25*g* for 5 minutes. Microarrays were scanned with the use of a GenePix 4000A microarray scanner with GenePix Pro 3.0 software (Axon Instruments, Union City, Calif); this yielded 2 16-bit images per array, 1 corresponding to the Cy3 channel and 1 corresponding to the Cy5 channel.

Data analysis. Image pairs were processed with the use of GenePix Pro 3.0 to obtain fluorescent intensity pairs, including background intensity, for each gene on each array. The data were normalized (within-print-tip-group loess location normalization with the use of all probes on each array) before log2 transformation, and plots were generated with the use of Bioconductor (www.bioconductor.org)[16] installed on the R software package.[17] The data were normalized to remove the effects of systemic variation, other than differential expression, in measured fluorescence intensities to correct for intensity, spatial, and other dye biases. Duplicate spots were averaged with the use of the SAS System (SAS Institute, Cary, N.C.). Significant changes in gene expression were