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Genetic Vulnerabilities to Prenatal Alcohol Exposure: Limb Defects in Sonic Hedgehog and *GLI2* Heterozygous Mice

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Abstract

Background—Genetic factors influence the physical and neurobehavioral manifestations of prenatal alcohol exposure (PAE). Animal models allow the investigation of specific genes that confer vulnerability to, or protection from, birth defects associated with fetal alcohol spectrum disorders (FASDs). The objective of the present experiments was to determine if genetic alterations in the Sonic Hedgehog (Shh) signaling pathways affect the vulnerability to PAE-induced skeletal defects involving the fore- and/or hind-limbs.

Method—Wild-type C57BL/6J female mice were bred with males in which one copy of the *Shh* or *Gli2* genes had been knocked out, to produce litters with both wild-type (+/+) and heterozygous (+/-) embryos. Alcohol doses (two injections of 2.9 g/kg, four hours apart) or vehicle were administered starting at GD-9.25, 9.5 or 9.75, a critical exposure time for inducing limb defects. Limb defects were examined at GD 17 using a dysmorphology scale based on abnormalities ranging from increased interdigital spacing to the deletion of multiple fingers and the ulna.

Results—Alcohol treatment caused a high incidence of forelimb defects, particularly on the right side, that was higher in *Shh*^{+/-} and *Gli2*^{+/-} fetuses compared to WT fetuses. Dysmorphology scores were also significantly higher in the *Shh*^{+/-} and *Gli2*^{+/-} mice.

Conclusions—These results extend previous findings demonstrating enhanced sensitivity to PAE-induced craniofacial dysmorphology and support the hypothesis that genetic alterations in the Shh signaling pathway influences the vulnerability to alcohol-induced birth defects. Moreover, these results emphasize the importance of understanding the interactions between genes and prenatal exposure to alcohol or other teratogens.

Keywords

Fetal Alcohol Syndrome; Limb Development; Oligodactyly; Ethanol; Skeletal

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Introduction

Genetic and environmental interactions are increasingly recognized as modifiers of many birth defects (Eberhart and Parnell, 2016; Krauss and Hong, 2016). In the case of prenatal alcohol exposure (PAE), one of the most common and preventable teratogen exposures, genetic risk factors may help explain the considerable variability in the consequences of PAE (Smith et al., 2014). Not all individuals exposed to alcohol prenatally develop the structural birth defects and/or neurobehavioral disturbances associated with fetal alcohol spectrum disorders (FASD) and those who do often express a range of defects, from relatively minor to severe. Although the amount and timing of PAE are clear factors in this variability, identifying the contribution of critical genes to the sensitivity to PAE is an important research agenda.

Many skeletal and limb defects have been reported in clinical populations with PAE, including radioulnar synostosis, campto- and clino-dactyly, ectrodactyly, polydactyly, and amelia (Herrmann et al., 1980; Pauli and Feldman, 1986; Spiegel et al., 1979). However, a recent large scale analysis showed that light patterns of alcohol exposure were not associated with limb defects, but that heavy patterns might be associated with a subset of severe defects (Caspers Conway et al., 2014). In animal studies, where the amount and timing of PAE can be carefully controlled, limb defects can be readily observed (Chernoff, 1977; Johnson et al., 2007; Kotch et al., 1992; McMechan et al., 2004) and, on the basis of strain comparisons, there appear to be genetic influences (Boehm et al., 1997; Zimmerman et al., 1990). In humans, several gene mutations are associated with limb defects (Zuniga et al., 2012) and it is possible that some of these may interact with PAE to make individuals more vulnerable to alcohol-induced limb defects.

Limb development is a carefully regulated and complex process that begins in embryos at the 20 – 30 somite stage (forelimb and hindlimb, respectively), in the fourth week of human pregnancy (equivalent to gestational days 9–10 in the mouse). Initial limb bud outgrowth from the flank mesenchyme and the formation of the apical ectodermal ridge (AER) is governed by the coordinated actions of retinoic acid (RA), fibroblast growth factors (FGF), bone morphogenetic proteins (BMP), WNT, and Hox genes. As outgrowth continues, AER FGFs and flank RA signals establish the Sonic Hedgehog (Shh) containing zone of polarizing activity (ZPA) in the caudal (posterior) limb bud. The temporal and concentration gradients of Shh as well as its downstream interactions with Gli transcription factors further determine the size of the developing limb and specify the number, and the type, of digits (Zeller et al., 2009). PAE during limb bud induction in the mouse causes a specific post-axial deficit that can resemble those of complete *Shh* gene deletions and Shh pathway inhibition (Litingtung et al., 2002). Remarkably, even in very severe alcohol-induced limb defects, where the entire ulna and digits 5, 4, and 3 are absent, the thumb and second digit are not affected, as these digits normally develop in the presence of little to no Shh (Johnson et al., 2007; Kotch et al., 1992).

Interference with Shh signaling is thought to be a pathogenic mechanism for several consequences of PAE, including craniofacial dysmorphology and limb defects (Ahlgren et al., 2002; Aoto et al., 2009; Johnson et al., 2007). Importantly, mice lacking a single copy of

the *CDON* (a Shh co-receptor) gene, the *Shh* gene, or its downstream activator *Gli2*, were more sensitive to alcohol-induced midline craniofacial defects (Hong and Krauss, 2012; Kietzman et al., 2014). These results highlight the fact that gene knockdowns that are not by themselves teratogenic, can enhance the vulnerability to PAE. The experiments described herein test the hypothesis that single allele deletions of *Shh* or *Gli2* would also enhance the vulnerability to alcohol-induced limb defects.

Methods

Animals

Dams (n = 75) were C57BL/6J mice (C57, The Jackson Laboratory, Bar Harbor, ME) mated to male *Shh*^{+/-} or *Gli2*^{+/-} mice (C57 mice in which one copy of the *Shh* or *Gli2* genes had been knocked out; Kietzman et al., 2014) for one to two hours to generate heterozygous and wild-type littermates. Detecting a copulatory plug established gestational day 0 (GD 0) at the beginning of the breeding period. Except for drug administrations, the dams were not disturbed until fetus collection. Food (Isopro RMH 3000; Purina, St. Louis, MO) and water were freely available and all experiments followed NIH guidelines using methods approved by the IACUC of UNC at Chapel Hill.

Procedures

Alcohol (25% v/v ethanol in lactated Ringer's solution) was injected intraperitoneally at a 2.9 g/kg dose starting at GD 9.25 (*Shh* = 13, *Gli2* = 12, litters), GD 9.5 (*Shh* = 10, *Gli2* = 8, litters), or GD 9.75 (*Shh* = 9, *Gli2* = 9, litters), and a second alcohol injection was given four hours later. Vehicle injected mice (*Shh* = 9, *Gli2* = 5, litters) received the equivalent volume of Ringer's. At GD 17, fetuses were collected, examined and scored for limb dysmorphology using the following scale (Figure 1): 0, unaffected; 1, slightly wide space between 4th and 5th digit; 2, wide space between 4th and 5th digit; 3, partial 5th digit deletion; 4, 5th digit deletion; 5, 4th digit deletion; 6, 4th and 5th digit deletion; 7, multiple digit deletion with apparent absence of the ulna. Genotyping was performed from a small sample of the tail (Kietzman et al., 2014). Fetuses were fixed in 95% ethanol for photographing and skeletal staining with an Alcian blue/Alizarin red procedure modified from McLeod (McLeod, 1980). After clearing in 1% KOH, forelimbs were photographed using a MicroPublisher 5.0 camera and QCapture Imaging software. The incidence of any right or left forelimb defect was compared between alcohol-treated heterozygous and wild-type mice using a Chi-square statistic for each alcohol exposure time. Unpaired t-tests compared the severity of limb defects among affected heterozygous and wild-type individuals, using the most severe defect on either the right or left forelimb. Dysmorphology scores (0 – 7) were further analyzed with two-way (Day x Genotype) ANOVAs.

Results

A total of 108 *Shh*^{+/-}, 130 *Shh*^{+/+}, 130 *Gli2*^{+/-}, 95 *Gli2*^{+/+} alcohol-treated and 28 *Shh*^{+/-}, 25 *Shh*^{+/+}, 21 *Gli2*^{+/-}, 17 *Gli2*^{+/+} vehicle-treated embryos were examined. No limb defects were observed in any of the vehicle-treated control or heterozygous mice (data not shown). However, post-axial, but not pre-axial, limb reduction defects (Figure 1) were observed

following each of the alcohol exposure times; these tended to be more frequent and severe after the GD 9.25 or 9.5 exposures, rather than the 9.75 exposures (Figure 2). Most defects were on the right forelimb, and to a lesser degree, the left forelimb; three hindlimb oligodactylies were observed in *Shh*^{+/-} mice after the GD 9.5 or GD 9.75 exposure. As previously reported (Kotch et al., 1992), there were occasional polydactylies; three hindlimb (one post-axial, two pre-axial) and three forelimb post-axial polydactylies were observed in *Shh*^{+/+} mice after the GD 9.5 exposure and one forelimb post-axial polydactyly was observed in a *Gli2*^{+/+} mouse after GD 9.25 exposure. Additionally, three GD 9.25-treated, one GD 9.75-treated *Gli2*^{+/-} mice, and one GD 9.75-treated *Gli*^{+/+} mouse had an extra cartilaginous nub attached to a digit or in one case, the presumed elbow (Figure 1, Defect 7). Polydactylies were not included in the analysis because of their low incidence.

Relative to *Shh*^{+/+} and *Gli2*^{+/+} mice, *Shh*^{+/-} and *Gli2*^{+/-} mice had a significantly greater incidence of limb defects following the GD 9.25 (*Shh* $\chi^2=9.5$; $p=0.002$, *Gli2* $\chi^2=7.0$; $p=0.008$) and 9.5 (*Shh* $\chi^2=8.6$; $p=0.003$, *Gli2* $\chi^2=6.2$; $p=0.01$) exposures (Figures 2A and 2B). Among affected individuals, *Shh*^{+/-} mice more severe defects following the GD 9.5 exposure ($t_{46}=2.3$; $p=0.03$), but the severity of affected mice was not significantly different between *Gli2*^{+/-} and *Gli2*^{+/+} mice. Analyzing the data using individual dysmorphology scores, indexing a combination of incidence and severity, there were significant main effects of *Shh* and *Gli2* genotype ($F_{1,232}=25.3$; $p<0.001$ and $F_{1,216}=20.8$; $p<0.001$, respectively), such that heterozygotes were more sensitive to alcohol-induced limb defects than were their wildtype littermates. Similar results were found when dysmorphology scores were analyzed using the genotype means from each litter. Surprisingly, the *Gli2*^{+/+} mice had a higher incidence of limb defects after GD 9.25 treatment, compared to the *Shh*^{+/+} mice and prior C57 studies. Since the *Shh*^{+/+} mice were most sensitive to GD 9.5 and GD 9.75 exposures, we hypothesized that *Gli2*^{+/-}-mated litters may have been developmentally advanced, as compared to the *Shh*^{+/-}-mated litters. However, a somite staging experiment revealed that *Gli2*^{+/-}-mated litters were only one somite pair older (2 hours) than were *Shh*^{+/-}-mated litters, indicating that embryo age does not entirely account for the difference. Further studies of alcohol sensitivity in *Gli2*^{+/-}-mated litters may uncover important paternally-derived contributions to PAE sensitivity.

Discussion

Many PAE effects on the brain, face, and limbs resemble those of genetic syndromes involving defects in Shh signaling, and alterations in this pathway may be critical pathophysiological mechanisms of PAE. The current study demonstrates forelimb defects in a mouse model following discrete PAE on the ninth gestational day (20–30 somite pairs, approximately the fourth gestational week in humans), confirming that limb bud induction is a sensitive exposure period for severe limb defects. Most importantly, the current study identifies a genetic vulnerability to alcohol-induced limb defects, as mice containing a single *Shh* or *Gli2* allele were more likely to develop limb defects than were their littermates with both copies of the genes. These results extend previous findings of Shh pathway genetic vulnerabilities to alcohol-induced craniofacial defects (Hong and Krauss, 2012; Kietzman et al., 2014) and reiterate the importance of understanding the complex interactions between alcohol and Shh.

Previous mouse studies have revealed that PAE during GD 9 induces excessive cell death in the AER and downregulates Shh expression in the ZPA, an effect that may be secondary to alcohol's hypothesized inhibition of RA synthesis, FGFs, or cholesterol modification (Chrisman et al., 2004; Duester, 1991; Johnson et al., 2007; Kotch et al., 1992; Li et al., 2007). Excessive cell death has not been noted in the ZPA, which suggests that, at least in the forelimb, PAE does not kill Shh expressing cells. In fact, Shh is not fully expressed in the C57 mouse limb bud until GD 10, a time when severe limb defects are very rare following PAE and likely occur only in the youngest stage embryos (Johnson et al., 2007).

Interestingly, the latest exposure period (GD 9.75), which would be expected to overlap with high levels of Shh expression, was the least sensitive to genotype influences, further suggesting that direct Shh inhibition is not the primary pathogenic mechanism of PAE. Rather, Shh pathway genetic vulnerabilities may reflect intermediate mechanisms between the initial PAE insult and the subsequent structural development of the limb. Alcohol undoubtedly affects multiple mechanisms in limb development, but the current results indicate that allelic variations in the Shh signaling pathway can alter genetic vulnerabilities to alcohol pathogenesis.

The presence of limb defects in clinical PAE populations may represent an important gene by environment interaction that should be studied further (May et al., 2010). Given the number of genes directly linked to limb defects (Zuniga et al., 2012), it is likely that many limb specific genes will interact with PAE. However, genes that affect Shh signaling, or mutations of the FGFs, BMPs, and WNTs, as well as their respective transcription factors, are strong potential candidate genes relevant for PAE-induced limb defects. There is significant conservation between the roles of Shh signaling in the development of the limb and craniofacies and brain; and converging evidence from both craniofacial and limb defects suggests that Shh signaling is a major modifier of PAE. Larger gene sequencing studies have so far focused on understanding the genetic contributions to craniofacial defects (Eberhart and Parnell, 2016). However, given the well-defined role of Shh in the limb, studying limb defects will be useful for understanding how PAE interacts with the Shh pathway. Furthermore, a genetic vulnerability involved in several PAE effects, such as craniofacial, limb dysmorphology, and neurobehavioral alterations, is likely to be one with a strong mechanistic role in FASD. Continued investigation of the interactions between Shh genotypes and PAE will help determine the full extent of the role of Shh signaling in FASD.

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Figure 1.

Dysmorphology scale for quantifying limb defects after alcohol treatment. *Top Panels:* Right forelimbs from GD 17 fetuses after fixation. *Bottom Panels:* The same forelimbs following Alcian Blue and Alizarin Red staining for cartilage and ossified bone, respectively. The *colored arrow* indicates the score for each defect ranging from 0 to 7: 0, unaffected; 1, slightly wide space between 4th and 5th digit; 2, wide space between 4th and 5th digit; 3, partial 5th digit deletion; 4, 5th digit deletion; 5, 4th digit deletion; 6, 4th and 5th digit deletion; 7, multiple digit deletion with apparent absence of the ulna. Note the appearance of an extra cartilaginous outgrowth in panel 7, *black arrow*.

Figure 2.

Incidence and severity of alcohol-induced limb defects. *Panels A. and B.* portray the incidence of either a left or a right forelimb defect (*filled bars*) following alcohol exposure on GD 9.25, GD 9.5, or GD 9.75 in *Shh*^{+/+} or *Shh*^{+/-} mice (*panel A.*) and in *Gli2*^{+/+} or *Gli2*^{+/-} mice (*panel B.*) The incidence of the most severe left or right forelimb defect is portrayed in colors graded from yellow (least affected, 1) to dark red (most affected, 7). Asterisks denote significant incidence differences between ^{+/+} and ^{+/-} mice following Chi square tests ($p < 0.05$). *Panels C. and D.* portray the average morphology scores ($\pm$ SEM, *vertical lines*) for each genotype following alcohol exposure (*Panel C.*: *Shh*^{+/+}, yellow circles; *Shh*^{+/-}, orange circles. *Panel D.*: *Gli2*^{+/+}, yellow triangles; *Gli2*^{+/-}, orange triangles). Asterisks denote significant morphology differences between ^{+/+} and ^{+/-} mice following a main effect of genotype ($p < 0.05$).