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Preaxial Polydactyly Following Early Gestational Exposure to the Smoothened Agonist, SAG, in C57BL/6J Mice

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Abstract

Background—While pharmacological activation of the Hedgehog (HH) signaling pathway may have therapeutic benefits for developmental and adult diseases, its teratogenic potential is of concern. The membrane molecule, Smoothened (SMO), transduces HH signaling and can be acutely modulated by antagonists and agonists. The objective of the current experiments was to determine how maternal treatment with the Smo agonist, SAG, affects the developing limb.

Methods—Pregnant C57BL/6J mice received a single injection of SAG (15, 17, or 20 mg/kg, i.p.) or its vehicle on gestational day (GD) 9.25, the time of limb bud induction. Embryos were examined on GD 15 for gross dysmorphology and skeletal staining was performed to visualize the number and type of digits on the fore- and hindlimbs. Additionally, *in situ* hybridization was performed four hours after GD 9.25 SAG administration to determine SAG's effects on *Gli1* and *Gli2* mRNA expression.

Results—The most prevalent effect of SAG was the dose-dependent induction of pre-axial polydactyly; defects ranged from a broad thumb to the duplication of two finger-like digits on the preaxial side of the thumb. The highest SAG dose was effective in ca. 80% of the embryos and increased *Gli1* and *Gli2* mRNA expression in the limb bud, with *Gli1* mRNA being the most upregulated.

Conclusion—Preaxial polydactyly can be caused in the developing embryo by acute maternal administration of a Smo agonist that activates HH signaling. These results are consistent with the preaxial polydactyly induced in developmental disorders associated with mutations in HH signaling genes.

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Keywords

Limb Development; Sonic Hedgehog; Polydactyly; Prenatal Exposure; Skeletal

Introduction

The hedgehog (HH) family of signaling molecules comprising Sonic (SHH), Indian (IHH), and Desert (DHH), is critical for developmental patterning, cell proliferation and cell survival (Pathi et al., 2001). Disrupted HH signaling through genetic mutation leads to birth defects including holoprosencephaly, cardiac, and limb malformations, while prolonged over activation of HH signaling is associated with basal cell carcinoma and medulloblastoma (Briscoe and Therond, 2013). Additionally, animal studies indicate that reduced SHH signaling is a teratogenic mechanism for compounds including veratrum derivatives and ethanol (Incardona et al., 1998; Johnson et al., 2007).

HH pathway modulation has therapeutic potential, including tumor suppression by antagonists and cellular protection by agonists (Ruat et al., 2014). Many of these agents act upon Smoothened (SMO), a seven-pass membrane protein typically repressed by Patched (PTC). HH enables SMO phosphorylation and shunting into the primary cilium where it activates downstream signaling involving GLI transcription factors and G-protein coupled receptors (Briscoe and Therond, 2013; Chen et al., 2002). Smoothened agonists such as SAG (i.e. SMO agonist), purmorphamine or the oxysterol nat-20(s)-OHC, might be predicted to ameliorate developmental syndromes resulting from reduced HH activity. Mouse studies have shown that postnatally SAG protects against glucocorticoid-induced neurotoxicity (Heine et al., 2011) and reverses cerebellar structural defects and some behavioral defects in models of Down Syndrome and stroke (Das et al., 2013; Gutierrez-Castellanos et al., 2013; Jin et al., 2015; Roper et al., 2006). In *Shh* deficient mouse embryos, SAG can rescue midline morphological and HH signaling defects in the anterior neural ridge and ventral neural tube (Frank-Kamenetsky et al., 2002).

SMO-acting compounds are also tools for developmental biology studies examining the effects of short-term HH signaling modulation. Cyclopamine has been studied extensively, with results confirming the temporally significant role of SHH in craniofacial and limb development (Incardona et al., 1998; Lipinski et al., 2014). Additionally, the SMO antagonist vismodigib can induce facial and palatal clefts as well as forelimb oligodactyly (Heyne et al., 2015).

Alternatively, agonist studies in *Xenopus*, have revealed increased HH signaling, calcium influx, cardiac dysmorphology, and a shortened body axis (Belgacem and Borodinsky, 2011; Lewis and Krieg, 2014). A study in chicks demonstrated that *in ovo* SAG can induce preaxial polydactyly in the wing (Johnson et al., 2014). However, there have been no reports in mice that a HH signaling agonist affects fetal morphology. The current study utilized the developing limb bud as a HH sensitive tissue to determine if maternal SAG administration can alter fetal morphology, consistent with HH signaling upregulation.

The limb bud is induced in mouse embryos having approximately 13-20 somite pairs; a stage present at approximately 9.25 gestational days (i.e. Theiler Stage 14-15). Signaling molecules including retinoic acid (RA) and fibroblast growth factors (FGFs) establish and maintain the zone of polarizing activity (ZPA) in the caudal (post-axial) aspect of the limb bud that expresses SHH in a spatial and temporal gradient. Cells exposed to the highest SHH concentration for the longest time become the ulna, digits 5 and 4, and a portion of digit 3. The remainder of digit 3 and all of digit 2 are determined by long-range SHH signaling, while the radius and digit 1 develop in the absence of SHH (Zeller et al., 2009). Reducing SHH signaling causes post-axial oligodactyly (Litingtung et al., 2002) while ectopic SHH expression duplicates digits (Riddle et al., 1993), as occurs in many genetic polydactylies (Anderson et al., 2012). The current hypothesis was that, consistent with HH upregulation, SAG would dose-dependently induce preaxial polydactyly when administered during limb bud induction.

Methods

Animals

Dams (n=28) were C57BL/6J mice (C57, The Jackson Laboratory, Bar Harbor, ME) mated to male C57 mice for one to two hours. Gestational day 0 (GD 0) was the beginning of the breeding period when a copulatory plug was detected. Dams were housed in groups of five or fewer in standard polycarbonate cages with cob bedding and cotton nesting material on the cage floor and food (Isopro RMH 3000; Purina, St. Louis, MO) and water freely available through the cage lid. Except for a single drug administration, the dams were undisturbed until embryo collection. All experiments followed NIH guidelines using methods approved by the IACUC of UNC at Chapel Hill.

Procedures

GD 9:6 hour females were weighed, given a single intraperitoneal SAG injection (N-methyl-n'-(3-pyridinylbenzyl)-n'-(3-chlorobenzo[b]thiophen-2-caronyl)-1,4-diaminocyclohexane, Santa Cruz Biotechnology, Dallas, TX; litters = 6, 6, and 7 for the 15, 17, and 20 mg/kg doses, respectively) or vehicle (lactated Ringer's solution; 9 litters), and returned to their home cage. GD 9:6 is a sensitive period for inducing forelimb malformations by retinoic acid and ethanol administration (Johnson et al., 2007). For SAG dose-response studies, GD 15 embryos were collected, examined for the number and appearance of the digits on each limb, fixed in Bouin's, and processed for Alcian blue skeletal staining (Nagy et al., 2009). For whole mount *in situ* hybridization studies, embryos were collected at GD 9:10 hours in ice-cold RNase-free phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA), rinsed with PBS, dehydrated in methanol and stored at -20 C.

Whole Mount *in situ* hybridization

Linearized template cDNA was isolated to make *Gli1* and *Gli2* *in vitro* transcription antisense probes using the T7 promoter. Plasmid DNA constructs were made using Bluescript vectors (Agilent Technologies) containing either mouse *Gli1* (plasmid Bluescript II SK (pBS)/mGli1) linearized with XbaI or mouse *Gli2* (pBS II KS/mGli2) linearized with HindIII. Linearized DNA was gel isolated, buffer exchanged and concentrated. DIG RNA

Labeling Kit (Roche) was used for *in vitro* transcription of cDNA to DIG-labelled antisense riboprobe. DNase I (Roche) was added to eliminate residual DNA template. Illustra ProbeQuant microcolumns (GE Healthcare) purified the riboprobe via centrifugation, and a Nanodrop (Thermo Scientific) determined the probe yield.

Embryos were bleached in 3% hydrogen peroxide in methanol and then rehydrated. Following digestion with Proteinase K (Roche), they were post-fixed with 4% PFA/0.2% glutaraldehyde in PBS/0.1% Tween-20 (PBT) and hybridized overnight at 65°C with the DIG-labeled riboprobes. After washing, treatment with RNase A/T1, and blocking, the sections were incubated overnight with an AP-conjugated anti-DIG antibody pre-absorbed with an embryo acetone powder to minimize non-specific binding. Blocking buffers and the anti-DIG antibody included levamisole (2mM, Sigma). Negative control experiments without anti-DIG antibody were carried out simultaneously. BM Purple AP Substrate (Roche) was utilized for chromogenic detection. The color reaction was stopped with EDTA; the sections were post-fixed, washed and photographed.

Results

Maternal administration of SAG caused dose-dependent dysmorphology, as observed in GD 15 fetuses, but did not affect the number of live or resorbed fetuses. Preaxial polydactyly was the most frequent defect, but mild edema and eye anomalies were also observed in a few fetuses (Figure 2A). In a separate preliminary study, these non-limb defects and especially wavy ribs were common and more severe when SAG was administered at GD 8.5, illustrating that as expected, embryo age at the time of exposure is important for the specific developmental effects of SAG. Figure 1 shows a spectrum of preaxial polydactylies; with defects ranging from the addition of a thumb-like process to the addition of two complete fingers. When the thumb was affected, it was typically broadened, bifurcated, or duplicated. Three fetuses had a thumb-like process attached to the second finger and one fetus had two extra finger-like digits with no apparent thumb. Single and double duplicated finger-like digits were positioned either before or after the thumb. The identity of the duplicated digits was not readily apparent, as the corresponding carpals were frequently indistinct. Figure 2A shows that the incidence of polydactyly depended on a narrow range of SAG doses between 15 and 20 mg/kg, with the highest dose being effective in approximately 80% of fetuses. In preliminary studies, no defects were observed at doses less than 15 mg/kg. As shown in Figure 2B, preaxial polydactylies were detected on each of the fore- and hindlimbs, but were most prevalent on the left forelimb, particularly after the 20 mg/kg dose.

Based on Heine et al., (2011) SAG activates GLI transcription factors, measured 4 hours after its administration. Therefore, we examined this time point using whole-mount *in situ* hybridization to visualize *Gli1* and *Gli2* mRNA expression 4 hours following SAG (20 mg/kg) or vehicle treatment. As shown in Figure 2C and 2D, SAG amplified the expression of *Gli1* mRNA and to a lesser extent *Gli2* mRNA. For *Gli1*, the expression pattern became distributed throughout the entire limb bud. For *Gli2*, which was already distributed throughout the limb bud, the expression was intensified and distributed into the proximal limb bud. (N.B. the left and right limb bud showed similar expression patterns, but only the right is shown in Figure 2C and 2D because its larger size enabled clearer visualization.)

Discussion

The current results demonstrate that acute pharmacological activation of HH signaling by the smoothened agonist SAG dose-dependently induces preaxial polydactylies in the mouse fore- and hindlimbs, extending results from a similar SAG exposure in chicken embryos (Johnson et al., 2014). Preaxial polydactylies occur in syndromes linked to modified HH signaling genes, such as Greig cephalopolysyndactyly, and in animal studies preaxial polydactylies are observed after constitutive down-regulation of GLI3 repression, or induction of HH signaling (Anderson et al., 2012). SAG stimulates the HH pathway *in vitro* (Chen et al., 2002), as we have replicated in a line of Hh responsive cells (Williams et al., unpublished), and the current results confirm that SAG can increase *Gli1* and *Gli2* mRNA expression in the mouse limb bud.

SAG-induced polydactyly primarily involved either broadening of the thumb/hallux or the addition of an indistinct ectopic finger-like digit. Postaxial defects were never noted, indicating that tissue that normally has the least SHH exposure was the most sensitive to SAG. The defect spectrum may be explained by embryo age differences at the time of SAG exposure. The oldest and youngest littermates can differ by up to 12 hours, with each embryo undergoing different limb development stages. The extra thumb-like digits may develop in the youngest embryos during limb induction by establishment of an ectopic, preaxial ZPA (Fig 2D) and an additional digit, as was shown in polydactylous *Silkie* and SAG-treated WT embryos (Johnson et al., 2014). As SAG levels decline, the ectopic SHH activation ceases and normal thumb-like digit specification occurs. In older embryos, SAG exposure may overlap with limb induction and digit specification enabling extra digits to develop into finger-like thumbs. Interestingly, SAG administrations to *Silkie* embryos inhibited lower leg polydactyly suggesting that specific SAG effects may depend on endogenous SHH activity (Johnson et al., 2014). SAG has biphasic effects on HH signaling *in vitro* (Frank-Kamenetsky et al., 2002) and it is possible that at higher SHH concentrations, SAG might inhibit HH signaling in the limb. Further gene expression studies at different times after SAG administration would help clarify SAG's actions in the limb.

SAG and other non-peptidic small molecule smoothened agonists have been proposed as pharmacotherapies for restoring HH signaling deficits. Animal studies using SAG postnatally have observed impressive responses with minimal toxicity. Heine et al. (2011) treated neonatal mice with SAG for one week and showed that SAG crossed the blood brain barrier and attenuated the neurotoxic effects of prednisone, but did not induce cerebellar tumors or other cancers, nor did it antagonize beneficial effects on lung maturation. Topical SAG (1.5 µg per day for 8 days) applied to 7-wk old mice promoted hair growth with minimal long-term effects on proliferation and differentiation up to 1 year later (Paladini et al., 2005). The potential of SAG to treat Down syndrome (DS)-related deficits has also been explored. A single SAG dose (20 mg/kg) on the day of birth rescued cognitive functions in a mouse model of DS (Roper et al., 2006) and this rescue was sustained in the adult (Das et al., 2013). However, postnatal SAG can affect craniofacial development depending on the dose (Singh et al., 2015). In a follow up study (Singh et al., 2016) comparing acute postnatal pharmacological- and chronic genetic-upregulation of Hh activity, varied and unpredicted effects on morphology were observed in different tissues. The authors concluded, as we have

herein, that Hh pathway stimulation does not represent a simple on/off developmental switch. The clinical utility of Hh stimulation may depend on when in development Hh becomes unregulated by a disease, as well as the magnitude and duration of the Hh stimulation. Given the potential teratogenic effects of SAG, extensive toxicity studies would be needed to optimize dose, schedule, and safety parameters prior to any clinical trials in humans, especially if SAG is given during early stages of pregnancy.

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Figure 1.

Alcian blue cartilage staining in representative forelimbs of embryos treated with SAG on GD 9.25. The *inset numbers* denote the total fore- and hind-limbs observed in each category, ranging from unaffected to thumb and multiple finger duplications. Note, the additional thumb growing off the second finger, shown in the second panel of the top row, was observed only on the right fore- and hind-limb; for consistency this picture has been flipped to the left side.

Figure 2.

Figure 2A and 2B SAG dose-response for observable defects in GD15 embryos. Figure 2C and 2D. *in situ* hybridization for *GLI* mRNA following 20 mg/kg SAG. 2A. Bars in the left panel represent the percent of mice showing polydactyly on at least one limb following GD 9.25 treatment with vehicle (open bar) or different doses of SAG (filled bars). Defects on single and multiple limbs are portrayed in light and dark gray, respectively. Bars in the center panel represent the percent of mice showing edema following different doses of SAG. Bars in the right panel represent the percent of mice showing an eye defect following different doses of SAG. Inset numbers denote the fetuses with defect/total fetuses treated. 2B. Bars represent the percent of polydactyly on the right and left fore- and hind-limbs after 15, 17, or 20 mg/kg SAG (left, center, and right panels, respectively). In each panel, left and right sided defects are displayed on the left and right sides of the dashed line. Inset numbers denote the fetuses with specific defect/total fetuses affected at that dose. Affected fetuses were observed in 2 of 6 litters treated with 15 mg/kg, 7 of 7 litters treated with 17 mg/kg, and 7 of 8 litters treated with 20 mg/kg. 2C and 2D display representative photographs (6x) of GD 9:10 embryos following *in situ* hybridization for *GLI1* (2C) and *GLI2* (2D) mRNA expression four hours after treatment with vehicle (left panels) or SAG (20 mg/kg, right panels).