Published in final edited form as:

Toxic Subst Mech. 2000 April ; 19(2): 125–133.

THE EFFECTS OF 5-AZA-2'-DEOXYCYTIDINE (D-AZA) ON SONIC HEDGEHOG EXPRESSION IN MOUSE EMBRYONIC LIMB BUDS

Stacy Branch

Department of Toxicology, North Carolina State University, Raleigh, North Carolina, USA

Ida W. Smoak

College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA

Abstract

5-Aza-2'-deoxycytidine (d-AZA) causes temporally-related defects in the mouse. At 1.0 mg/kg on gestational day (GD) 10, d-AZA causes hindlimb phocomelia. Sonic hedgehog (Shh) plays a significant role in the normal development of limbs in rodent species. Sonic hedgehog peptides, found in the posterior mesenchyme of limb buds, are involved in patterning functions and in the regulation of both anterior-posterior polarity and proximal-distal outgrowth of the limb. The objective of the present study was to analyze alterations in Shh expression subsequent to d-AZA exposure. Pregnant mice were treated with d-AZA via intraperitoneal injection on GD 10. Controls were untreated. The reverse transcription-polymerase chain reaction (RT-PCR), whole mount in situ hybridization (ISH), and whole mount immunohistochemistry (WMI) were used to analyze expression patterns of Shh. For RT-PCR, embryonic hindlimb buds (buds) were taken 0, 4, 8, 12, or 24 hr after exposure. Cyclophilin was used as the baseline monitor. RNA was transcribed to cDNA and used as template with Shh specific primers for amplification. Whole embryos were collected 12 and 24 hr posttreatment for ISH. An antisense primer specific for Shh was used in an oligo-based ISH protocol. Whole embryos were collected 36 and 48 hr posttreatment for WMI. The antibody corresponding to the amino terminal subunit of the Shh peptide was used. There was a treatment related up-regulation of Shh transcripts by 12 and 24 hr posttreatment. The protein response of up-regulation was detectable by 36 and 48 hr posttreatment. Our data suggest that 5-aza-2'-deoxycytidine-induced hindlimb defects may be associated with alterations in the level of Shh expression. This may be part of a cascade of signaling events involved in d-AZA-induced hindlimb defects. Work is ongoing to determine the relationship of other gene species that may cooperate with Shh in the induction of the hindlimb defects.

The sonic hedgehog (*Shh*) gene encodes peptide signals involved in vertebrate limb patterning. It is associated with patterning of the anterior-posterior (A-P) axis of the developing limb and mediates the activity of the zone of polarizing activity (ZPA). In addition to anterior-posterior patterning of the developing limb, *Shh* is also necessary for the continued outgrowth of the limb (Nakamura et al., 1997; Scott, 1997; Chiang et al., 1996). The interactions of sonic hedgehog (within the ZPA) with apical ectodermal ridge (AER) help to establish patterning of all three of the limb axes (Johnson et al., 1994; Tickle & Eichele, 1994; Martin, 1995).

In chicks, the AER factor Fgf-4 can be induced by ectopic expression of *Shh* (Laufer et al., 1994). Riddle et al. (1993) demonstrated that retinoic acid-soaked beads implanted into the anterior margins of host limb buds induced ectopic expression of *Shh*. Implanting *Shh*-

Address correspondence to Stacy Branch, North Carolina State University, Department of Toxicology, Method Road, Unit 4, Box 7633, Raleigh, NC 27695, USA. E-mail: Stacy_Branch@ncsu.edu.

This work was supported by NIH # ES08452, National Institute of Environmental Health Sciences.

expressing chick embryo fibroblasts to host limb buds induces limb duplications that resemble those induced by ZPA grafts. Implanted *Shh*-expressing cells in the anterior region of stage 21 chick limb buds lead to duplications of the radius and digits II and III (Vortkamp et al., 1996). Limb buds of *Xt/Xt* mice (that exhibit disturbed A-P patterning) have an additional domain of *Shh* expression when compared with wild-type mice. This second domain persists through gestational day (GD) 12, whereas normal *Shh* expression peaks at approximately GD 11 and decreases later. Further, *Fgf-4* expression was extended in the mutant mice. This suggests that *Shh* is the major activity in the ZPA (Büscher et al., 1997; Ingham, 1995).

5-Aza-2'-deoxycytidine (d-AZA) causes the passive removal of methyl groups from DNA due to the prevention of its binding to the 5 position of cytidine residues. Treatment of CD-1 mice with this compound causes temporally-related developmental defects (Branch et al., 1996). At 1.0 mg/kg on GD 10, d-AZA causes hindlimb phocomelia. Similar defects are not detected in the forelimb when treating with d-AZA at the similar stage of development, suggesting that the hindlimb defects are not due to cytotoxicity alone. Since d-AZA may exert its biological effects by altering gene expression, studies examining expression of *Shh* were conducted. The objective was to determine whether altered expression of *Shh* is associated with long bone defects elicited by d-AZA.

MATERIALS AND METHODS

Animals

To reduce interlitter variation in staging, CD-1 mice (Harlan Farms or Charles River) were bred from 12 am to 2 am. Animals with plugs were included in the study. The time (9 am) after plug identification was considered day 0.

Dosing and Tissue Collection

Mice were treated with 1.0 mg/kg 5-aza-2'-deoxycytidine at 9 am on GD 10. Fore and hind limb buds (buds) were collected 0, 4, 8, 12, or 24 hr after dosing. Buds of three litters per time point were pooled to use the litter as the experimental unit. Whole embryos were collected 12 and 24 hr posttreatment for use in whole mount in situ hybridization (ISH) and 36 and 48 hr posttreatment for whole mount immunohistochemistry WMI. Embryos for WMI were collected 36 or 48 hr after treatment to account for time necessary for the protein levels to change in response to changes in transcript levels. Buds and embryos to be used for reverse transcription-polymerase chain reaction (RT-PCR), and ISH were collected in RNase-free phosphate buffered saline (PBS) and stored at $-^{\circ}\text{C}$, while embryos for WMI were collected in sterile PBS.

Reverse Transcription-Polymerase Chain Reaction

The semiquantitative RT-PCR studies described below were performed to assess relative mRNA levels (Branch et al., 1998; Covert & Splitter, 1995; Rottman et al., 1995; Jakubowski et al., 1991). The Pre-amplification Kit (Gibco BRL, Bockville, Md) was utilized for reverse transcription of RNA using oligo dT primer. An oligo (dT) primer was used for the cDNA synthesis. Treated or untreated total RNA was added to oligo (dT) primer, PCR buffer, dNTP mix, and dithiothreitol as provided by the preamplification kit. Then, 10% of the cDNA product was used as a template for the PCR reactions, each of which was done in duplicate. A master mix containing enough cDNA, PCR buffer (Stratagene, La Jolla, CA), and dNTPs for 4 PCR reactions was prepared. Of these reactions 2 contained the primer corresponding to the studied *Shh*, while the remaining reactions contained the primer for the internal standard, cyclophilin, used as a baseline monitor. Cyclophilin amplification served to ensure that different samples contained similar amounts of RNA. It also demonstrated that the reverse-transcription reactions proceeded with equal efficiency. The master mix approach decreased sources of variation

between PCR reactions. Serial dilutions of template were prepared to verify that detection occurred in the linear range of amplification. The primers for cyclophilin and *Shh* were designed using the Primer3 program:

- Cyclophilin: forward primer 5'CTGGACCAAACACAAACG 3'; reverse primer 5'AATTAGAGCTGTCCACAG 3'.
- *Shh*: forward primer 5' GAAGATCACAAGAAACTCCGAACG 3'; reverse primer 5' TGGATTCATAGTAGACCCAGTCGAA 3'.
- PCR: A preliminary cycle of PCR was done (94°C—2 min, 60°C—1 min, 72°C—1 min) followed by 31 additional cycles of (94°C—30 sec, 60°C—1 min, 72°C—1 min) followed by a final cycle (94°C—30 sec, 60°C—1 min, 72°C—10 min).

The PCR products were separated on a 1.6% agarose gel and analyzed by densitometry (IP LabGel documentation system). The average values were used to compare band densities:

$$\text{Average Value} = (I_{xy} / N) - B$$

I_{xy} = pixel values within the region; B = the background value; N = number of pixels in the region. Differences between the control and treated samples were tested for significance using the Student's *t*-test.

Whole Mount In Situ Hybridization

Whole embryos were collected in RNase-free 10X PBS buffer then fixed in RNase-free 4% paraformaldehyde. After fixative removal, embryos were washed, twice with PBT (1X PBS with 0.1% tween). Embryos were then dehydrated sequentially using 25%–100% methanol followed by storage at –20°C overnight.

Embryonic heads were removed to facilitate probe penetration. The following oligonucleotide protocol was based on a report by Gibco BRL (El-Badry & Darfler, 1998). Adaptations were implemented for the use of whole embryo tissue:

Embryos were rehydrated (75–25% v/v methanol/PBT–PBS with tween) at room temperature, then rinsed three times with PBT. Embryos were transferred to 2 ml screw-cap tubes (2 embryos per tube) and treated with 5 µg/ml of proteinase K for 10 min at room temperature. Embryos were fixed in fresh 0.2% glutaraldehyde/4% paraformaldehyde in PBS at room temperature for 20 min, then rinsed three times with PBT. Embryos were then treated with 0.1% sodium borohydride in PBT for 20 min in uncapped tubes followed by rinsing twice with hybridization buffer (25% formamide, 10% dextran sulfate, 2X SSC), then prehybridized for 1 hr. Hybridization buffer was replaced and 1 µl biotin-labeled oligo was added for overnight hybridization at 25°C. Embryos were rinsed and incubated in blocking solution (Gibco BRL) for 15 min, followed by covering embryos with working conjugate (Gibco BRL). Embryos were washed in alkaline-substrate buffer (100 mM tris base, 150 mM NaCl, and 50 mM magnesium chloride-hydrate) followed by addition of NBT/BCIP and incubated until desired signal (~30 min).

Reactions were stopped using several changes of deionized water. Embryos were then dehydrated and stored in 4:1 glycerol/PBTE with 0.02% Na Azide (4°C). Antisense oligonucleotide sonic hedgehog oligo probe:

5' TGGATTCATAGTAGACCCAGTCGAA 3'.

Whole Mount Immunohistochemistry

Embryos were collected in 1X PBS and fixed in 4% paraformaldehyde for 48 hr. The heads and tails were cut to improve tissue exposure to antibodies. Embryos were dehydrated in 70–

100% methanol in 1X PBS at room temperature (30 min per methanol concentration). Endogenous peroxidases were blocked by incubation for 2 hr in 3% H₂O₂ in 100% methanol at room temperature. Embryos were then stored in 100% methanol at -20°C overnight. Embryos were rehydrated in 90–70% methanol in 1X PBS at room temperature then placed in 1X PBS for 30 min at room temperature. Embryos were placed in PBS/tween with 1% block (nonfat dried milk) and primary antibody (1:10 goat antimouse sonic hedgehog polyclonal; Research Diagnostics, Flanders, NJ) overnight at 4°C. Controls were incubated in PBS/tween with 1% block. Embryos were washed five times with PBS/tween for 1 hr each at room temperature. Embryos were then incubated in PBS/tween with 1% block and secondary antibody (1:200 rabbit antigoat IgG horseradish-peroxidase conjugate; Zymed, San Francisco, CA) overnight at 4°C. Immunoperoxidase staining was performed with 4-chloro-1-naphthol solution for 30 min at room temperature until a color change appeared. Embryos were then washed with 1X PBS in a Petri dish and photographs taken.

RESULTS

Expected hindlimb longbone defects (phocomelia and reduced or absent tibiae and fibulae) (Branch et al., 1996) were observed in mouse fetuses allowed to remain in utero until GD 17. RT-PCR produced a *Shh* product represented by a band of approximately 300 base pairs (cyclophilin produced a band of ~200 base pairs). There was a treatment-related twofold up-regulation of *Shh* in hindlimb bud tissue detectable via RT-PCR by 12 hr posttreatment ($p < 0.05$) and a 2.5-fold increase detectable by 24 hr posttreatment, $p < 0.05$ (Figure 1). Changes in expression were not detected in hindlimb buds collected at earlier time points, and no change (treated vs. control) in *Shh* expression was detectable in the forelimb bud tissues (not shown). No positional misexpression was detectable by ISH nor were quantitative differences visible using this technique (not shown). *Shh* protein is sparse at these stages, but the higher concentrations are observed in limb buds and heart tissue. Visible increases in *Shh* protein were observed in the limb buds of treated embryos by 36 and 48 hr posttreatment (Figure 2).

DISCUSSION

Strict breeding windows were necessary to minimize interlitter variation in stage-specific levels of *Shh* expression. A twofold or more difference in the RNA expression of *Shh* was detectable by RT-PCR. This degree of difference was difficult to detect using whole mount in situ hybridization. Since the differences seen with RT-PCR were observed after amplification of the signal, such differences may be difficult to detect by a non-PCR method. However, one could still assess presence of ectopic or altered anatomical expression. Anatomical misexpression did not accompany the quantitative change observed. The corresponding protein levels were readily visible. As with other patterning genes (Hox genes), minor changes in transcript abundance are difficult to detect by in situ hybridization, but changes of only twofold in the level of patterning proteins can lead to morphologic transformations (Andrew & Scott, 1992).

Previous protein analysis studies to detect *Shh* protein in embryos collected at stages earlier than 36 hr posttreatment did not reveal changes in *Shh* protein levels. When using buds collected at least 12 hr after observing the strongest up-regulation of transcript, changes in corresponding protein levels were detectable. Using whole mount immunohistochemistry, a minor increase in *Shh* protein was observed in the buds of embryos collected 36 hr after treatment; however, the increase was stronger by 48 hr posttreatment.

Work by Akiyama et al. (1997) suggests that signalling molecules such as *Shh* are involved in chondrogenesis and cartilage differentiation. Alterations of signaling pathways involving these molecules may result in the abnormal phenotypes associated with the d-AZA-induced hindlimb

defects. Although *Shh* is primarily associated with antero-posterior patterning, it also has a role in proximodistal axis patterning (Niswander et al., 1993; Johnson et al., 1994) due to its regulatory relationship with fibroblast growth factor-4. Therefore, it is feasible that alterations in *Shh* expression may be associated with longbone reduction defects. Since changes in *Shh* expression were not detectable in the forelimb tissues, the lack of d-AZA-induced forelimb defects may be associated with differences in the methylation patterns of fore- and hindlimb gene species that regulate *Shh* and longbone outgrowth.

Treated embryos are smaller than the corresponding controls. This and the observation of increased *Shh* expression may be a consequence of delayed development induced by the treatment. As mentioned, *Shh* expression usually peaks at GD 11 followed by decreased expression. As seen in the WMI embryos, *Shh* expression is barely detectable in normal embryonic GD 11.5 (36 hr posttreatment) and GD 12 (48 hr posttreatment) buds. However, the same-stage treated embryos have higher expression levels. These embryos may express *Shh* at a level found at an earlier stage embryo. This delay may still alter the timing of patterning events leading to the abnormal phenotype. It is also feasible that it is a response to an earlier change in expression of other regulatory genes.

This laboratory has found that the expression of other patterning genes (homeobox genes) is affected by d-AZA treatment at GD 10. Hox a-11 and c-11 are down-regulated. Interestingly, the expression of Hox d-11 is not altered (unpublished). Sonic hedgehog can inhibit its receptor patched and patched has been found to inhibit the transcription of Hox genes (Scott, 1997). Sonic hedgehog also has been shown to activate hox genes (Riddle, 1993). This up-regulation in *Shh* may be a response to the Hox a and c-11 down-regulation and may influence the expression level of Hox d-11, thus preventing abnormal down-regulation of this homolog. Preliminary studies in our laboratory examined the effects of treatment on other genes (bone morphogenic protein [*Bmp4*] and fibroblastic growth factor 4 [*fgf4*]) that cooperate with or are influenced by *Shh*. We did not reveal changes in expression levels of the genes by RT-PCR earlier than 36 hr posttreatment. Further work is ongoing to determine the effects on these and other genes influenced by sonic hedgehog (such as patched, smoothened). Other work involves determining the role of altered methylation in the altered expression of developmentally relevant genes to further understand molecular disruptions leading to xenobiotically induced abnormal phenotypes.

REFERENCES

- Akiyama H, Shigeno C, Hiraki Y, Shukunami C, Kohno H, Akagi M, Konishi J, Nakamura T. Cloning of a mouse smoothened cDNA and expression patterns of hedgehog signalling molecules during chondrogenesis and cartilage differentiation in clonal mouse EC cells, ATDC5. *Biochem. Biophys. Res. Commun* 1997;235(1):142–147. [PubMed: 9196051]
- Andrew D, Scott MP. Downstream of the homeotic genes. *The New Biologist* 1992;4:5–15. [PubMed: 1346973]
- Branch S, Francis BM, Brownie CF, Chernoff N. Teratogenic effects of the demethylating agent 5-aza-2'-deoxycytidine in the Swiss Webster mouse. *Toxicology* 1996;112:37–43. [PubMed: 8792847]
- Branch S, Francis BM, Rosen MB, Brownie, Held GA, Chernoff N. Differentially expressed genes associated with 5-aza-2'-deoxycytidine-induced hindlimb defects in the Swiss Webster mouse. *J. Biochem. Mol. Toxicol* 1998;12:135–141. [PubMed: 9522272]
- Büscher D, Bosse B, Heymer J, Rüther U. Evidence for genetic control of Sonic Hedgehog by Gli3 in mouse limb development. *Mech. Dev* 1997;62:175–182. [PubMed: 9152009]
- Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 1996;383:407–413. [PubMed: 8837770]

- Covert J, Splitter G. Detection of cytokine transcriptional profiles from bovine peripheral blood mononuclear cells and CD4+ lymphocytes by reverse transcriptase polymerase chain reaction. *Vet. Immunol. Immunopathol* 1995;49:39–50. [PubMed: 8588343]
- El-Badry OM, Darfler MM. Evaluation of biotinylated oligonucleotide probes for in situ hybridization. *Focus* 1998;18:70–72.
- Ingham PW. Signalling by hedgehog family proteins in *Drosophila* and vertebrate development. *Curr. Opin. Genet. Dev* 1995;5:492–498. [PubMed: 7580142]
- Jakubowski M, Blum M, Roberts JL. Post-natal development of gonotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain. *Endocrinology* 1991;128:2701–2708.
- Johnson RL, Riddle RD, Laufer E, Tabin C. Sonic hedgehog: a key mediator of anterior-posterior patterning of the limb and dorso-ventral patterning of axial embryonic structures. *Development* 1994;111:45–60.
- Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C. Sonic hedgehog and Fgf-4 act through a signalling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 1994;79:993–1003. [PubMed: 8001146]
- Martin GR. Why thumbs are up. *Nature* 1995;374:410–411. [PubMed: 7700347]
- Nakamura T, Aikawa T, Iwamoto-Enomot M, Iwamoto M, Higuchi Y, Maurizio P, Kinto N, Yamaguchi A, Noji S, Kurisu K, Matsuya T. Induction of osteogenic differentiation by hedgehog proteins. *Biochem. Biophys. Res. Common* 1997;237:465–469.
- Niswander L, Tickle C, Vogel A, Booth I, Marting GR. Fgf-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* 1993;75:579–587. [PubMed: 8221896]
- Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 1993;75:1401–1416. [PubMed: 8269518]
- Rottman JB, Freeman EB, Tonkonogy S, Tompkins MB. A reverse transcription-polymerase chain reaction technique to detect feline cytokine genes. *Vet. Immunol Immunopathol* 1995;45:1–18. [PubMed: 7604528]
- Scott NP. Hox genes, arms and man. *Nature Gene* 1997;15:117–118.
- Tickle C, Eichele G. Vertebrate limb development. *Ann. Rev. Cell Biol* 1994;10:121–152. [PubMed: 7888174]
- Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin C. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 1996;273:613–622. [PubMed: 8662546]

Figure 1.

Representative agarose electrophoresis gels (1.6%) of hindlimb RT-PCR products. Differential expression of *Shh* in 12 hr (A) and 24 hr hindlimb tissue (B). M-molecular weight marker, 1,2-treated duplicate sample (*Shh* related); 3,4-control duplicate sample (*Shh* related); 5,6-treated duplicate sample (cyclophilin related); 7,8-control duplicate sample (cyclophilin related). Cyclophilin internal standard is not altered by the treatment. (C) Optical density data from gels (A) and (B) plotted as the mean of the O.D. ratios of duplicates $\pm$ standard error; T-treated; C-untreated control.

Figure 2.

Representative whole mount immunohistochemistry. Staining of GD 11.5 (36 hr posttreatment) and GD 12 (48 hr posttreatment) whole embryos exposed to *Shh* primary antibody. Black arrows designate hindlimb buds. There is an increase in *Shh* protein in the limb bud of treated animals collected 36 hr posttreatment (A) compared with the corresponding controls (B). The increase in *Shh* protein of animals collected 48 hr posttreatment is more pronounced by 48 hr posttreatment (C). (D)-corresponding 48 hr posttreatment control. Staining in forelimbs of all specimen is low or not visible.