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Determinants of orofacial clefting I: Effects of 5-Aza-2′-deoxycytidine on cellular processes and gene expression during development of the first branchial arch

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ABSTRACT

In this study, we identify gene targets and cellular events mediating the teratogenic action(s) of 5-Aza-2′-deoxycytidine (AzaD), an inhibitor of DNA methylation, on secondary palate development. Exposure of pregnant mice (on gestation day (GD) 9.5) to AzaD for 12 h resulted in the complete penetrance of cleft palate (CP) in fetuses. Analysis of cells of the embryonic first branchial arch (1-BA), in fetuses exposed to AzaD, revealed: 1) significant alteration in expression of genes encoding several morphogenetic factors, cell cycle inhibitors and regulators of apoptosis; 2) a decrease in cell proliferation; and, 3) an increase in apoptosis. Pyrosequencing of selected genes, displaying pronounced differential expression in AzaD-exposed 1-BAs, failed to reveal significant alterations in CpG methylation levels in their putative promoters or gene bodies. CpG methylation analysis suggested that the effects of AzaD on gene expression were likely indirect.

1. Introduction

In the United States approximately 6800 babies are born annually with orofacial clefts, such as cleft lip with or without cleft palate (CL/P), and isolated cleft palate (CP) [1,2]. Without proper treatment, such orofacial anomalies often result in feeding and speech impediments, dental abnormalities, hearing loss, ear infections and breathing difficulties. In addition, the affected individuals, owing to their appearance, endure diverse social, emotional and psychological challenges [3].

Palatal clefting primarily occurs as the result of anomalous morphogenesis of the secondary palate. The maxillomandibular prominences of the first branchial arch (1-BA) give rise to various structures of the embryonic orofacial region (e.g. maxilla, mandible, and lateral portions of upper lip) including the palatal processes, precursors of the secondary palate or roof of the oral cavity [4]. The embryology of this process is similar in humans and mice. The critical events include emergence of the bilateral palatal processes as oral projections from the maxillary prominences, reorientation of these processes from a vertical position, lateral to the tongue, to a horizontal position above the tongue, and finally, fusion of these processes to each other along their anterior–posterior length forming the definite secondary palate [5].

Morphogenesis of the orofacial region (including the secondary palate) is reliant, in part, on the migration of neural crest cells derived from the neuroectoderm of rhombomeres 1–3 [6] into the first two branchial arches, and diversification of neural crest cell fates [7]. Since the 1-BAs serve as the palatal precursor tissue, precise regulation of expression of genes and the downstream cellular processes they guide within these tissues is essential for normal development of the secondary palate. Numerous studies using

Abbreviations: aCasp3, anti-activated caspase 3; aPH3, anti-phosphohistone H3; 1-BA, first branchial arch; AzaD, 5-Aza-2′-deoxycytidine; CGI, CpG island; CP, cleft palate; CL/P, cleft lip with or without cleft palate; DAPI, 4′-6′-diamidino-2-phenylindole; FBS, fetal bovine serum; GD, gestational day; IPA, Ingenuity Pathway Analysis; MCS, methylCap-sequencing; NSCLP, non syndromic cleft lip and cleft palate; PFA, paraformaldehyde; qRT-PCR, quantitative real-time PCR; TLDA, Taqman low-density arrays.

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animal models have documented that anomalous 1-BA development results in CP [8–12]. Indeed, altered Wnt [11] or Shh [13] signaling in the 1-BA, both pivotal for normal development of the 1-BA, results in CP. Similarly, defects in development of the 1-BAs are also thought to contribute to craniofacial dysmorphologies that accompany Apert, Pierre Robin and Treacher Collins syndromes [14,15].

Numerous environmental factors such as nutritional deficiencies, exposure to cigarette smoke, alcohol, drugs, pesticides, and microbial infection, have been implicated in the etiology of orofacial dysmorphologies [16–21]. This allows for the hypothesis that a potential epigenetic mechanism by which some of these environmental factors elicit tissue dysmorphology is aberrant gene methylation. Support for such a notion comes from documentation of an epigenetic role of the *clf2* gene in the multifactorial etiology of cleft lip and palate [22], and significant CpG island- and global DNA hypomethylation during retinoic acid-induced CP [23].

Epigenetic processes alter gene expression during embryogenesis and mediate heritable activation or silencing of genes in specific cell lineages [24–26] without modifying DNA sequences. Such processes include, but are not limited to, DNA methylation, microRNA (miRNA) function, and histone modification. Mammalian DNA methylation, occurring predominantly at cytosine residues within CpG dinucleotides, typically regulates the expression of specific genes through a transcriptional repression process catalyzed by a family of active DNA methyltransferases (DNMTs) and transcriptional regulators, collectively known as methyl-CpG-binding proteins [27,28]. The cytidine analogue, 5-Aza-2′-deoxycytidine (AzaD), is a potent DNA methylation inhibitor, and teratogen. By forming covalent adducts with cellular DNMT1, AzaD-substituted DNA inhibits DNMT1 activity and triggers demethylation of genomic DNA [29,30].

AzaD has been extensively employed to investigate the role of DNA methylation in regulating gene expression during embryogenesis [31–35]. AzaD treatment of murine fetuses on GD 9.0 has been reported to induce palatal clefting [36,37]. The current studies examining presumptive epigenetic and cellular mechanisms underlying the pathogenesis of AzaD-induced CP, identified key genes encoding several morphogenetic factors, signaling mediators, cell cycle inhibitors and pro-apoptotic factors targeted by AzaD in the developing 1-BAs.

2. Materials and methods

2.1. Animals

Mouse (ICR strain) husbandry and developmental staging of embryos was conducted as described previously [38]. All procedures for the humane use and handling of mice were approved by the University of Louisville Institutional Animal Care and Use Committee and encompass guidelines as set out in the European Commission Directive 86/609/EEC for animal experimentation.

2.2. 5-Aza-2′-deoxycytidine dosing regimen and tissue collection

5-Aza-2′-deoxycytidine was obtained from Sigma Chemical Co., St. Louis, MO. AzaD dosage (1 mg/kg; [37]) was based on the average dam weight on GD 9.5 and was administered in sterile PBS solution. Control embryos were dosed with an equal volume of sterile PBS (vehicle). AzaD or vehicle was delivered via single intraperitoneal injection to pregnant females on GD 9.5 and embryos were collected on GD-15.5 and -17.5. For individual TaqMan quantitative real-time PCR (qRT-PCR) and for TaqMan array card-based gene expression profiling, 1-BAs of AzaD- or vehicle exposed embryos were microdissected 6, 9 and 12 h post-exposure and pooled for analyses (Fig. 6). These three treatment periods were selected on the basis of preliminary data (TaqMan qRT-PCR and immunocytochemistry) that demonstrated: (1) significant, temporal, and differential expression of several genes, and (2) a striking temporal decrease in cell proliferation and marked increase in apoptosis within tissue of the 1-BA. 1-BA tissue from three independent pools of 12–15 staged embryos was collected, total RNA and genomic DNA extracted (AllPrep DNA/RNA Mini Kit; Qiagen Inc., Valencia, CA), and stored at −80 °C. Whole embryos were also collected 6, 9 and 12 h post-treatment, fixed in 4% paraformaldehyde (PFA), embedded in OCT (optimal cutting temperature compound, Tissue-Tek 4583, Sakura Finetek USA, Inc., Torrance, CA), frozen at −80 °C, cryosectioned and processed as described by Oh et al. [39], either for staining with hematoxylin and eosin, or immunostaining with phospho-histone-H3 or activated caspase-3 antibody, to assess cell proliferation or apoptosis, respectively.

2.3. "Fetal phenotyping" and identification of palate phenotype

On GD 15.5 and 17.5, dams exposed to AzaD or vehicle, were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Uterine horns were exteriorized, and the number and location of implantations and resorptions were recorded. Fetuses were removed from the uterus, blotted, weighed, measured for crown–rump length, and inspected for gross external anomalies. Palatal development and/or degree of palate fusion was assessed by removing the lower jaw for direct visualization of the palate.

2.4. Immunohistochemistry

Embryos exposed *in utero* (on GD 9.5 for 6, 9 and 12 h) to either AzaD (1 mg/kg) or vehicle, were fixed in 4% PFA, washed in PBS, cryoprotected in sucrose (5–10% in PBS), incubated overnight at 4 °C in 20% sucrose, immersed and embedded in OCT. Eight μm coronal sections of the embryos were cut using a Leica CM1900 cryostat (Leica Inc., Bannockburn, IL) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Tissue sections were washed, permeabilized in 0.1% Tween 20, and blocked in 0.1% Tween 20/1.0% BSA/5% FBS (in PBS) for 30 min at 37 °C. Sections were immunostained overnight at 4 °C with a 1:500 dilution of rabbit anti-phospho Histone H3 (Upstate-Millipore, Billerica, MA) for evaluation of proliferation, or rabbit anti-activated caspase 3 (Cell Signaling Technology Inc., Danvers, MA) for evaluation of apoptosis. Sections were then washed in PBS and goat anti-rabbit AF-594 secondary antibody (Molecular Probes – Life Technologies, Grand Island, NY) at a 1:200 dilution and blocking solution was applied to the sections for 60 min at 37 °C. Cellular DNA was counterstained with 300 nM 4′-6′-diamidino-2-phenylindole (DAPI) (Molecular Probes – Life Technologies) and mounted under Fluoromount-G (Southern Biotech, Birmingham, Alabama) to preserve fluorescence. Tissue sections were visualized and photographed with a Nikon Eclipse TE 2000-U microscope equipped with epifluorescence optics. Fluorescent signal intensity was measured using the MetaMorph 6.5 software (Universal Imaging Inc., Downingtown, PA). To assess proliferation and apoptosis in the 1-BAs, the integrated intensity of fluorescence was measured (within the same viewing field using the MetaMorph software) utilizing triplicate sections per embryo. These experiments were performed using independent and comparable sections (in triplicate) derived from AzaD – or vehicle-exposed embryos from each treatment group (6, 9 and 12 h).