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Faulty bone morphogenetic protein signaling in esophageal atresia with tracheoesophageal fistula

Amanda R. Crowley, Sheilendra S. Mehta, Mark J. Hembree, Barry L. Preuett, Krishna L. Prasadan, Susan W. Sharp, Hooi Yew, Christopher R. McFall, Christina L. Benjes, Sidhartha S. Tulachan, George K. Gittes, Charles L. Snyder*

Laboratory for Surgical Organogenesis, Department of Surgery, Children's Mercy Hospital, Kansas City, MO 64108, USA

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Abstract

Background: The organogenesis of esophageal atresia with tracheoesophageal fistula remains unclear. We have previously demonstrated that the fistula tract develops from a trifurcation of the embryonic lung bud and displays pulmonary lineage traits. Unlike the lung, the fistula grows without branching. Bone morphogenetic proteins (BMPs) are known to be important in lung branching. We studied possible BMP signaling defects as a potential cause for the absence of branching in the fistula tract.

Methods: Adriamycin was administered to pregnant rats on days 6-9 of gestation to induce tracheoesophageal fistula. Microdissection was performed at E13 and E17 isolating the foregut. Tissues were analyzed using immunohistochemistry for BMP ligand (BMP2, BMP4, BMP7) and receptor (BMPRIA, BMPRIB, BMPRII) expression.

Results: Immunohistochemistry revealed the presence of all 3 BMP ligands at E13, localized specifically to the esophageal mucosa but absent in the fistula and lung. At E17, the ligands were again present in the esophageal mucosa, and additionally in the fistula tract mucosa, but remained absent in the lung. At E17, all of the BMP receptors were also localized to the luminal surface of esophagus and fistula. However, in the lung epithelium, only BMPRII was found, whereas BMPRIA and BMPRIB remained absent.

Conclusions: The normal expression pattern of BMP4 was increased at the branch tips and low between branches. Among other results, we show here a constant expression level of BMP ligands throughout the entire epithelium of the fistula tract. This diffuse expression suggests defective BMP signaling in the fistula tract and explains its nonbranching phenotype.

The mechanism of formation of esophageal atresia with tracheoesophageal fistula (EA/TEF) remains unclear. This anomaly, which occurs in approximately 1 in 3000 human births, has been mimicked in a rodent model of adriamycin-induced TEF, providing a way to study it developmentally [1-3]. We have shown that the fistula tract develops from a trifurcation of the embryonic lung bud, where the fistula tract grows without branching, whereas the 2 bronchi branch normally. The fistula bud continues to grow distally and fistulizes with the stomach

* Corresponding author. Tel.: +1 816 234 3576; fax: +1 816 983 6885.
E-mail address: csnyder@cmh.edu (C.L. Snyder).

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on embryonic day (E) 12.5 [4,5]. Although the fistula appears histologically to be a "distal esophagus," the functional and genetic phenotype of the fistula is "respiratory-like," which is consistent with its origin from the embryonic lung bud trifurcation. In patients with EA/TEF, the distal esophagus demonstrates poor motility and contains regions of pseudostratified columnar epithelium; which may indicate a respiratory origin of this structure [4,6]. It is likely that the distal fistula then undergoes metaplasia, transforming the histology to squamous epithelium seen in the normal gastrointestinal tract. Respiratory epithelium in the fistula is also seen early in development in the rat model. In addition, genetic analysis of foregut specific transcription factors has further confirmed a respiratory origin of the fistula tract in the rat model. Specifically, thyroid transcription factor (TTF-1), which is normally expressed only in the respiratory tract, was found in the airway as well as the developing fistula tract, but not in the stomach, providing more evidence of the respiratory origin of the fistula tract [7-9].

If the fistula is actually a nonbranching respiratory derivative, defects in bone morphogenetic protein (BMP) signaling may play a role in the pathogenesis of EA/TEF because BMPs are crucial to normal lung branching. However, the exact role of BMPs in lung branching morphogenesis is difficult to determine because many of the BMP signaling knockout mice die early in gestation before foregut formation [10]. Thus, transgenic animals have been used to better study the role BMP signaling plays in branching. Overexpression of BMP4 driven by the SP-C promoter/enhancer causes abnormal lung morphogenesis with smaller lungs at midgestation (E15.5) and less epithelial branching with greatly distended terminal buds. This phenotype was more pronounced at E18.5, and at birth, large air-filled sacs were observed, which did not support normal lung function [11]. Inhibition of BMP signaling by overexpressing the BMP antagonist noggin, and a dominant negative form of BMP receptor IB using the SP-C promoter have also been examined. Overexpression of either of these transgenes results in reduction of distal epithelial cell types but increases proximal cell types. Inhibition of BMP signaling both at the ligand level with noggin or at the receptor level has a deleterious effect on lung morphogenesis, although the exact role of BMPs has yet to be determined [12].

In this study, we used the adriamycin rat model to examine expression patterns of BMPs and their receptors in the development of EA/TEF.

1. Materials and methods

1.1. Animals

Time-dated pregnant Sprague-Dawley rats (day 0.5 corresponding with noon of the day of discovery of a vaginal plug) were obtained from Charles River Laboratories (Wilmington, Mass). Experimental rats were injected intraperitoneally with 2.0 mg/kg of doxorubicin hydrochloride (adriamycin) (Bedford Laboratories, Bedford, OH) on days 6-9 of gestation. Control rats were injected intraperitoneally with normal saline on days 6-9 of gestation. Euthanasia was performed by carbon dioxide inhalation–induced narcosis followed by cervical dislocation in accordance with approved protocol from the Institutional Animal Care and Use Committee of our institution.

1.2. Tissue preparation

Embryos from experimental and control animals were harvested on gestational days 13 and 17 and preserved on ice in Dulbecco Modified Eagle Medium for either tissue or cDNA preparation. Microdissection was performed on each embryo, isolating the TEF anomaly, including trachea, lung buds, fistula, and stomach. Microdissection of control embryos included collection of trachea, lung buds, esophagus, and stomach. Isolated TEF and control tissues were kept on ice in Dulbecco Modified Eagle Medium until the entire litter was dissected. Fresh 4% paraformaldehyde was prepared in phosphate-buffered saline (PBS), and tissues were fixed for 2 hours at 4 °C. The tissues were then transferred to 30% sucrose at 4 °C overnight. Tissues were finally embedded and frozen with Tris buffered saline freezing medium for cryosectioning.

1.3. Polymerase chain reaction

The tissue was processed for reverse transcriptase polymerase chain reaction (RT-PCR) using the Qiagen RNeasy MiniKit (Valencia, Calif) followed by Promega RQ1 DNase treatment (Madison, Wis) and the Qiagen Sensiscript RT kit (Valencia, Calif). The cDNA was amplified using the following primers: BMP2, 5′ gtttggcctgaagcagagac and 3′ gaagttcctcgatggcttct; BMP4, 5′ ggaggaggaggaagaagagc and 3′ gggatgctgctgaggttaaa; BMP7, 5′ caaccatgctatcgtccaga and 3′ aggatgacgttggagctgtc; BMPRIA, 5′ ggctgatcctcagtggaaat and 3′ tgtcttcacaagttagggacca; BMPRIB, 5′ gctttggactcatcctctgg and 3′ cctcataagaagggtcactgg; BMPRII, 5′ aaagcccagaagagcacaga and 3′cctgatcctgatttgccatc. PCR conditions included an annealing temperature of 58°C and 40 cycles.

1.4. Immunohistochemistry

Sections from both control and experimental groups were subjected to the same protocol for immunohistochemical detection of BMP ligands and receptors using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif). Slides were washed in 0.5% Tween for 10 minutes and 1× PBS for 5 minutes initially and between each subsequent step. Antigen retrieval was accomplished with 50 mmol glycine, pH 3.5, for 30 minutes at 60 °C. Slides were then blocked with 1.5% normal rabbit serum in PBS for 1 hour