Sonic Hedgehog and Bone Morphogenetic Protein 4 Expressions in the Hindgut Region of Murine Embryo With Anorectal Malformations

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Purpose: The aim of this study was to determine the possible role of the retinoid-mediated signaling pathway in the pathogenesis of anorectal malformations (ARM). The authors investigated whether all-trans retinoic acid (ATRA) affects the expression pattern of Sonic hedgehog (Shh) and Bone morphogenetic protein 4 (BMP4), which play important roles in anorectal morphogenesis in vertebrates.

Methods: Pregnant ICR strain mice were fed 100 mg/kg of ATRA on the ninth gestational day (E9). Embryos with or without administration of ATRA were obtained from the uteri between E12 and E16 and were fixed immediately in a 4% paraformaldehyde solution. Frozen sections were evaluated for concentric layers around the endodermal epithelium by H&E and immunohistochemistry using antibodies created specifically to act against Shh and BMP4.

Results: More than 95% of the embryos administered ATRA had ARM; rectoprostatic urethral fistula, rectocloacal fistula, and short tail were the most frequent anomalies in the mouse embryos. On E14, normal mouse embryos had normal rectum and anus in which the epithelium of the anorectum was positive for Shh, and the mesenchyme was positive for BMP4. In the ARM embryos, however, the epithelium of the anorectum was negative for Shh, and the mesenchyme was also negative for BMP4.

Conclusions: In normal hindgut development, Shh from the epithelium induces BMP4 expression in the mesenchyme, which differentiates into the lamina propria and the submucosa. In ARM embryos, expressions of Shh and BMP4 could not be found in those regions of the hindgut. Therefore, these findings indicate that Shh and BMP4, which appear to play a crucial role in organogenesis of the hindgut, were disturbed in the cell signaling pathway between the epithelium and the mesenchyme layers.

INDEX WORDS: Retinoic acid, sonic hedgehog, bone morphogenic protein 4.

ANORECTAL MALFORMATIONS (ARM) are the most common abnormality of the neonatal digestive system, occuring in approximately 1 in 5,000 live births.[1] The pathogenesis of ARM remains poorly understood.

In normal hindgut development, Sonic hedgehog gene is well known to play a critical role in the first phase of hindgut morphogenesis.[2-4] Shh mutants were reported to show tracheoesophageal malformations, intestinal transformation of stomach, duodenal stenosis (obstruction), abnormal innervation of the gut, and imperforate anus.[5-9] Its protein product (Shh) is thought to be an inductive signal acting on the adjacent visceral mesoderm, inducing bone morphogenetic protein 4 (BMP4) and activating *Hox* genes within the mesoderm.[10-12] BMP4 is known to act as a signal in an epithelial-mesenchymal interaction with Shh in the early phase of hindgut formation, and *Hox* genes are known to be involved in patterning of the vertebrate hindgut.[13]

The authors have previously reported that exposure of murine embryos to teratogenic doses of all-trans retinoic acid (ATRA) induced hydronephrosis and vertebral and anorectal malformations.[14,15] We also reported evidence that the overdose of ATRA affects the distal hindgut development by directly disrupting the retinoid mediated signaling pathway by the histologic evidence of the impaired distribution pattern of retinoic acid receptors (RARs) in ARM embryos.[16,17]

It had not been investigated whether overdose of ATRA could disturb the expression of Shh and BMP4 in the early phase of hindgut morphogenesis.This study investigated the pattern of Shh and BMP4 expression using ATRA-treated mouse embryos to elucidate the pathogenesis of ARM.

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MATERIALS AND METHODS

The ICR mice were obtained from Japan SLC Inc and were maintained in our colony. The rooms were kept on a 12-hour light-dark cycle with a constant temperature range. One male mouse and 3 female mice were kept together for several hours. At the end of the period, the presence of a vaginal plug was considered that the mating was successful and was regarded as day 0 of gestation (E0). On E9.0, pregnant mice were fed 100 mg/kg of ATRA suspended in sesame oil by intragastric gavage. Embryos were removed from the uteri between E12 and E16 using a dissecting microscope, Model SMZ-U (Nikon, Tokyo, Japan). Embryonic specimens were fixed immediately in 4% paraformaldehyde solution in phosphate buffer overnight at 4°C. Specimens then were cryoprotected by immersion in 30% sucrose in PBS for 24 hours and embedded in OCT compound (Tissue-Tek 4583; Sakura Finetek USA Inc, Torrance, CA) before freezing with liquid carbon dioxide. Sagittal serial sections (16 μm in thickness) were cut on a cryostat and mounted on coated glass slides.

Immunohistochemistry to detect Shh and BMP4

The tissue blocks were cryoprotected in 30% sucrose in phosphate buffer saline (PBS) and frozen in liquid carbon dioxide. Sagittal serial sections (16 μm) were cut on a cryostat and mounted on glass slides. The sections were pretreated with 0.3% hydrogen peroxide diluted in PBS for 30 minutes at room temperature. These specimens then were incubated in 1.5% blocking serum in PBS to block nonspecific staining followed by incubation with primary antibody directed against Sonic hedge hog (Shh) and bone morphogenetic protein 4 (BMP4) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature. The antibody concentration was 5 μL/mL, diluted in 1.5% blocking serum in PBS. Specimens also were incubated for 30 minutes with biotinylated secondary antibody (75 μL normal blocking serum, 5 mL PBS and 25 μL biotinylated secondary antibody), then incubated with avidin and biotinylated HRP mixing enzyme reagent for 30 minutes. Finally, the specimens were incubated in peroxidase substrate until the stain intensity was definitively developed.

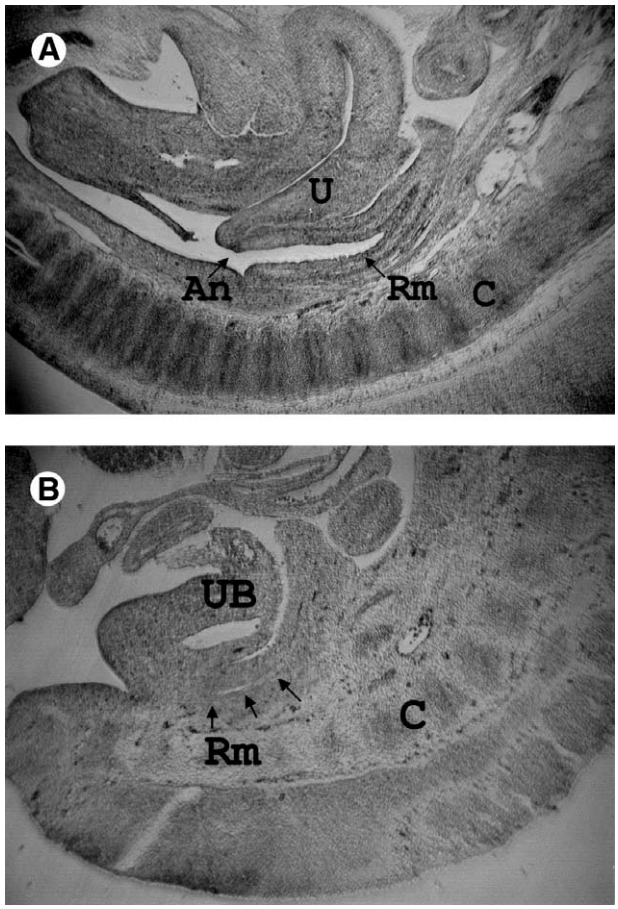

Fig 1. (A) Immunohistochemical study of Shh in midsagittal sections of ICR murine embryo on E13. Immunoreactivity for Shh was found in the epithelium of the rectum, urinary bladder, and urethra. (B) Immunohistochemical study for Shh in midsagittal sections of the ATRA-treated embryo on E13. Immunoreactivity for Shh was absent in the epithelium of the rectum, (arrow) urinary bladder and urethra (DAB, original magnification ×200). A, anus; Rm, rectal mucosa; U, urethra; UB, urinary bladder.

RESULTS

Among the ATRA-treated fetuses, 2% to 5% embryos died in utero. The overall survival rate of fetuses was greater than 95%, and all embryos had short tail and imperforate anus in which rectoprostatic urethral fistulas and rectocloacal fistulae were the most frequent anomalies in males and females, respectively.

Early in rectal development, largely distinct domains of expression of Shh were established in normal controls. Immunoreactivity specific to Shh was detected most abundantly in the hematopoietic megakaryocytes and the epithelium of anus, rectum, urinary bladder, and urethra. The signal pattern for Shh was also observed in the cartilaginous mesenchyme and the neural tube (Fig 1A). The abundant immunoreactivity for BMP4 was localized in the mesenchyme directly beneath the epithelium of the anus and rectum. As for the epithelium of the urinary bladder and urethra, the signal specific for BMP4 was positive but slightly weak compared with those in the anorectal region. The signal pattern for BMP4 was also observed in the cartilaginous mesenchyme and the neural tube (Fig 2A). In the affected ATRA-treated embryos, the immunoreactivity specific to Shh was detected in the hematopoietic megakaryocytes, cartilaginous mesenchyme, and neural tube. However, the signal for Shh was absent in the epithelium of the anus, rectum, urinary bladder, and urethra (Fig 1B). The immunoreactivity specific to BMP4 was also detected in the hematopoietic megakaryocytes, cartilaginous mesenchyme, and neural tube. However, it was not detected in the mesenchyme underlying the epithelium of the anus, rectum, urinary bladder, and urethra (Fig 2B). From these histologic findings, teratogenic doses of ATRA remarkably disturbed Shh and BMP4 expression in restricted regions of the urinary tract and hindgut on E14.

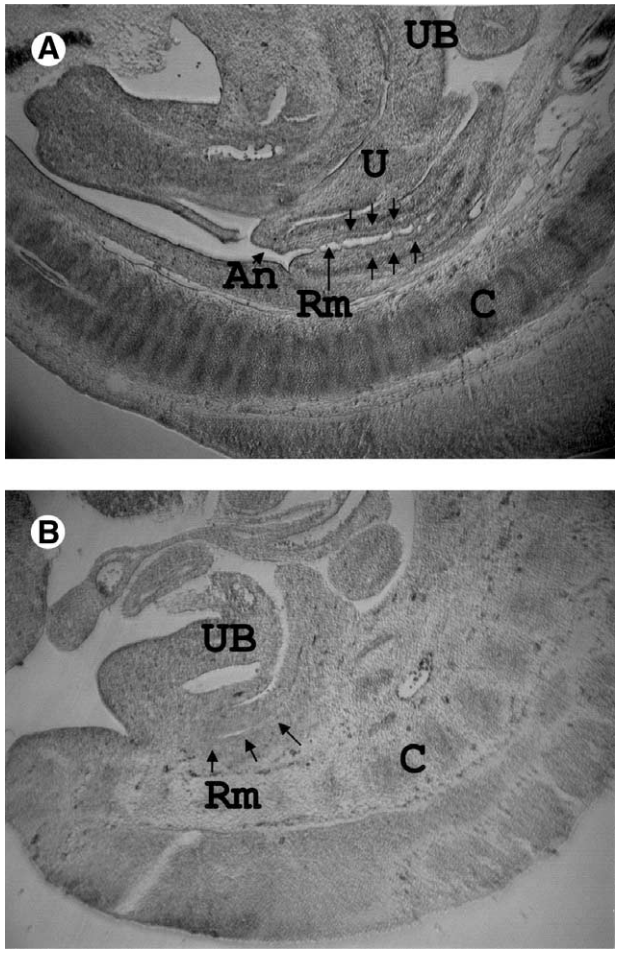

Fig 2. (A) Immunohistochemical study for BMP4 in midsagittal sections of ICR murine embryo on E13. Immunoreactivity for BMP4 was found in the epithelium of the rectum, urinary bladder, and urethra. BMP4 was also detected in a thin layer of mesenchyme directly beneath the epithelium. (B) Immunohistochemical study for BMP4 in midsagittal sections of the ATRA-treated embryo on E13. Immunoreactivity for BMP4 was absent in the epithelium of the rectum, urinary bladder, and urethra. BMP4 was also negative in a thin layer of mesenchyme directly beneath the epithelium (arrow DAB, original magnification ×200). A, anus; Rm, rectal mucosa; U, urethra; UB, urinary bladder.

DISCUSSION

This study showed that teratogenic doses of ATRA disturbed the expressions of both Shh and BMP4 in urinary tract and hindgut region of ATRA-treated embryos with ARM. Retinoic acid is known to be an essential factor for normal development in many organs.[18] Recent molecular biologic studies have shown also that retinoid-mediated signal transduction plays a critical role in the embryogenesis of various organs by regulating expression of *Hox* genes.[13,19] Retinoic acid is also known as a teratogen, and exposure of its teratogenic doses can produce changes in *Hox* gene expression and causes a wide range of structural congenital malformations, including anorectal malformations, in relation to the dosage and timing of exposure.[20-26]

Shh is known to be a key factor in gut morphogenesis and is expressed in the definitive endoderm in the early embryonic areas in which gut formation begins, at the anterior and posterior end of the embryo.[3,20,27] Around Shh expression, the *Hox* genes are expressed in a nested pattern in the pregut mesoderm.[12] Endodermally derived Shh normally functions to induce mesodermal BMP4 expression, implied to be a secondary signal in an inductive cascade, during formation of the gut tube.[13] Primitive gut endoderm is capable of signaling underlying mesoderm to induce visceral mesodermal differentiation.[10] This evidence was provided by an experiment using Shh mutant mice. Shh and its downstream gene mutant mice exhibit a spectrum of distal hindgut defects mimicking human anorectal malformations such as persistent cloaca, recto-urethral fistula, and anal stenosis.

Although, in our model, anorectal malformation was induced not by Shh mutation but by maternal administration of teratogenic doses of ATRA, we were very interested in the correlation between the retinoic acid overdose and Shh and its downstream gene product BMP4, the key substances for the early hindgut morphogenesis. Surprisingly, that gene expression was diminished in the hindgut region on E14. The result of this study can not deny the possibility that direct interference from teratogenic doses of ATRA on the activation of *Hox* genes is one of the important causes in the pathogenesis of ARM. However, we speculate that disturbance in the expression of Shh and BMP4 in the hindgut region could be the main cause of the pathogenesis of ARM in our model because Shh is the upstream regulator of *Hox* genes.

We also showed that Shh and BMP4 expressions were disturbed not only in the hindgut region but also in the urinary tract epithelium and adjacent mesenchyme. This is interesting evidence because many children with ARM also show urinary tract anomalies. To date, such anomalies were explained by incomplete partitioning of the cloaca, which may lead to the formation of rectourethral or rectocloacal fistula. However, our results show that the urinary bladder and urethra may undergo the same pathologic process described as an epithelial-mesenchymal interaction with Shh and BMP4 as seen in hindgut region in this model.

This study provides evidence that overdose of ATRA disturbed Shh and BMP4 expressions in both the urinary tract and hindgut regions. Further analysis of the cascade reaction important for hindgut morphogenesis should facilitate an understanding of the pathogenesis of ARM in humans.

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Discussion

P. Tam (Hong Kong, China): Can sonic hedgehog and BMP 4 rescue the ATRA-treated mice? Have you done experiments to establish a cause and effect relationship by giving the ARM mice sonic hedgehog or BMP 4 to rescue the mice from the deformity?

K. Kimura (response): We have not performed the rescue programme for this experiment, but it would be very interesting to pursue.

P. Kim (Toronto, Ontario): The hedgehog, retinoic acids and the BMP 4 are fairly general morphogens affecting deveopment from the top to the bottom. Do you have any speculation as to what restricts the development of this particular phenotype into that particular region in terms of reciprocal interaction between epithelium and mesenchyme?

K. Kimura (response): That is a very important point. In this presentation, we focused on the hindgut and its morphogenesis. However, we also actually noticed that the expression of Shh and BMP 4 was also negative in the duodenum. From this finding, we speculate that overdose of retinoic acid affects development not only in the hindgut region but also in the foregut and the midgut in terms of reciprocal interaction between epithelium and mesenchyme. But we have not carried out further analysis in this area yet.