Animal Model

Embryonic Gut Anomalies in a Mouse Model of Retinoic Acid-Induced Caudal Regression Syndrome

Delayed Gut Looping, Rudimentary Cecum, and Anorectal Anomalies

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Vitamin A and its derivatives such as retinoic acid (RA) are important signaling molecules for morphogenesis of vertebrate embryos. Little is known, however, about morphogenetic factors controlling the development of the gastrointestinal tract and RA is likely to be involved. In the mouse, teratogenic doses of RA cause truncation of the embryonic caudal body axis that parallel the caudal regression syndrome as described in humans. These changes are often associated with anomalies of the lower digestive tract. Overlapping spatiotemporal expression of retinoic acid receptor- β (RAR β) and cellular retinol-binding protein I, CRBPI, with Hoxb5 and c-ret in the gut mesoderm imply possible cooperation required for proper neuromuscular development. To determine susceptibility and responsiveness of the developing gut and its neuromusculature to exogenous retinoids we used a mouse model of RA-induced caudal regression syndrome. The results showed that stage-specific RA treatment both *in vivo* and *in vitro* affected gut looping/rotation morphogenesis and growth of asymmetrical structures such as the cecum together with delayed differentiation of the gut mesoderm and colonization of the postcecal gut by neural crest-derived enteric neuronal precursors. These observations demonstrate that RA has a direct effect on gut morphogenesis and innervation. (Am J Pathol 2001, 159:2321–2329)

Anomalies of enteric ganglia and smooth muscles are found in various congenital digestive tract malformations such as atresia, stenosis, anorectal anomalies, or bowel malrotations. Molecular mechanisms underlying these abnormalities are primarily unknown but disturbance in early gut morphogenesis may be implicated.¹ Migration, colonization, and subsequent maturation of the precursors of the enteric nervous system depend on the existence of preformed gut structures and trophic factors released from developing muscle layers.^{2,3} An early disturbance in gut morphogenesis and colonization by neural crest cell precursors because of abnormal cell migration, extracellular matrix, or cell-cell adhesion may produce a number of intestinal malformations involving enteric neurons and muscles.¹ Little is known about morphogenetic factors controlling gastrointestinal tract development, however, retinoic acid (RA) signaling may be involved. Retinoids regulate cellular growth and differentiation through direct or indirect transcriptional activation of target genes via RA-responding elements (RAREs) and RA receptors (RARs, RXR).⁴ Pharmacological administration of RA leads to a variety of malformations that depend on the dosage and developmental stage of the exposure. In particular, increased doses of RA, administered at specific gestational stages, result in hamster, rat, and mouse embryos in lumbar-sacral truncation of the body axis. This is often associated with lower digestive tract abnormalities such as imperforate rectum or intes-

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tinal atresia.^{5–8} RA-induced intestinal malformations in murine embryos resemble anomalies resulting from either vitamin A deficiency (VAD) or combined null mutations in the retinoic acid receptor that result in malformations of visceral organs including the lower and upper digestive tract.^{9,10} Interestingly, all of these intestinal anomalies are found in the spectrum of congenital abnormalities described in humans such as caudal regression syndrome, VATER, VACTERL, or Currarino triads.^{11,12} Similar defects produced by gene disruption, dietary deprivation, or excess of retinoids indicate that biologically active retinoids are essential for similar developmental programs reflecting normal retinoid-dependent processes that further support the involvement of RA signaling in gut morphogenesis. Defects in such an important signaling pathway or in its regulatory components could be a factor in a proportion of congenital gut malformations. During gut development, *RAR β* and *CRBP1* expression domain¹³ are localized in peripheral gut mesenchyme and coincide spatially and temporally with the reported expression domains of neuronal and mesenchymal differentiation markers, such as *c-ret*, *Hoxb5*, and α -smooth muscle actin (α -SMA).¹⁴ Such overlapping expression domains suggest cooperation of these genes that could be important for enteric neuromuscular development. Studying RA-induced caudal agenesis in murine embryo can represent a useful experimental model to investigate gut responsiveness to exogenous RA. It may also give an insight into molecular pathogenesis of intestinal malformations such as gut malrotations, atresia, and stenosis often coexisting with enteric neuromuscular anomalies described in humans.^{15,16}

Materials and Methods

Experimental Strategy

The influence of exogenous RA *in vivo* and in cultured gut explants was studied using *in situ* hybridization and immunohistochemistry for markers of neuronal and mesenchymal development, *c-ret*, *Hoxb5*, and α -SMA. The presence of apoptotic cells in embryos and dissected guts after *in vivo* and *in vitro* RA treatment was also monitored with colorimetric terminal transferase-mediated dUTP nick-end labeling (TUNEL) method.

Injections of Pregnant CD1 Mice with RA

Pregnant females from CD1 (Charles River Mouse Farms, Margate, Kent, UK) crossings from natural overnight matings were used. The morning of the vaginal plug was considered as 0.5 days post coitum (dpc). All-trans RA (Fluka, Poole, Dorset, UK) was dissolved in dimethyl sulfoxide (DMSO) as 100 mg/ml of stock solution and kept in the dark at -20°C until use for up to 1 week. Before injection, RA stock solution was diluted in DMSO and dispersed by vortexing in 1 ml of peanut oil. The RA dose and stage of injection sufficient to induce truncation of the caudal body axis and agenesis of the tail bud in embryos were selected following a previously described protocol.⁷

In four separate experiments, four pregnant mice were injected intraperitoneally with 120 mg/kg of RA (Fluka, Gillingham, Poole, Dorset, UK) at 9.5 dpc that coincides with the onset of gut morphogenesis.¹⁷ A total of 39 embryos were harvested at E10.5 ($n = 9$), E11.5 ($n = 12$), E12.5 ($n = 10$), and E16.5 ($n = 8$) at a similar time point in the morning into ice-cold diethyl pyrocarbonate-treated phosphate-buffered saline (DEPC/PBS), preserved by incubating overnight in 4% paraformaldehyde in PBS and dehydrated through the methanol/PBS grades into absolute methanol and stored at -20°C until use. Four control mice were injected intraperitoneally with DMSO dispersed in peanut oil and 32 embryos were harvested at the same time points as those in the RA-treated group E10.5 ($n = 8$), E11.5 ($n = 10$), E12.5 ($n = 8$), and E16.5 ($n = 6$). Gut development was assessed by the appearance of gut looping and the cecal bud that *in vivo* occurs between E11.5 and E12.5.¹⁴ The expression of neural and muscular markers such as *c-ret*, *Hoxb5*, and α -SMA served to assess differentiation as previously described.^{14,17,18–20}

In Vitro Culture of Gut Explants

Pregnant mice were sacrificed by spinal dislocation at similar time points in the morning at E10.5 and embryos removed into ice-cold L-15 Leibowitz medium (Life Technologies Ltd., Paisley, UK). The culture system was established as described previously.¹⁷ To parallel *in vivo* conditions, the earliest stage of the E10.5 guts consisting of a simple, straight tube was chosen as suitable for intact dissection. Guts were aseptically isolated from embryos and placed in pairs into wells of 24-well culture dishes with 500 μl of defined, serum-free, Optimem medium (Life Technologies Ltd.) supplemented with 1 mmol/L L-glutamine and L-amino acids with addition of 100 U/ml of antibiotic mixture (Life Technologies, Ltd). Intestines were kept floating in the medium and incubated for 3 days at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in air. The development and viability of gut explants was assessed according to the criteria used for embryos harvested from RA-treated mice such as the appearance of gut loops and the cecal bud and by the expression pattern of neural and muscular markers.

Retinoic Acid Treatment of Gut Explants in Culture

In the preliminary experiments, the dose of RA sufficient to induce morphological changes in gut explants consistent with those observed *in vivo* was established. The toxic effect of RA was assessed by the appearance of apoptotic cells in gut explants using the TUNEL method (see below). A total of 38 gut explants representing the youngest developmental stage of the gut appearing as a simple unlooped tube available for intact isolation at the age of 10.5 dpc were dissected and cultured for 24 hours in RA-free medium. After checking the explants viability manifest by a visible growth of the prececal gut or at-

tached lung buds, sets of 8 to 10 explants were chosen and cultured with increasing concentrations of RA: 10^{-7} mol/L ($n = 10$), 10^{-6} mol/L ($n = 10$), 10^{-5} mol/L ($n = 10$), and 10^{-4} mol/L ($n = 8$). The next day, RA medium was removed, the explants washed twice in fresh medium and cultured for the next 24 hours in RA-free conditions. At the end of 72 hours in culture, explants were washed in ice-cold PBS and preserved in 4% paraformaldehyde for 20 minutes. The lowest RA concentration (10^{-5} mol/L) that induced morphological changes in gut looping and neuromuscular development in the majority of tested explants comparable to those observed after *in vivo* treatment, but without the toxic effect manifest by the appearance of cell death, was chosen for the following experiments: to investigate the expression pattern of α -SMA, Hoxb5, and *c-ret* in morphologically altered gut explants. Gut explants dissected at E10.5 were cultured for 24 hours in RA-free medium. The explant viability was examined on the next day as described above. A total of 60 explants were chosen for further culture. Thirty gut explants were RA-treated and the same number of control guts was cultured in the absence of RA with the addition of the volume equivalent of DMSO as vehicle and control. Gut explants were placed in the fresh medium with the addition of 10^{-5} mol/L RA dilution from 100 mg/ml of stock solution in DMSO and explants were cultured for another 24 hours.²¹ The next day RA media were removed, explants washed twice with fresh media, and cultured for further 24 hours. At the end of culturing, explants were washed in ice-cold PBS for 15 minutes and preserved in 4% paraformaldehyde in PBS.

Detection of Apoptotic Cells

Whole embryos, dissected guts, or gut explants were assessed for the presence of apoptotic cells. TUNEL with Dead-End colorimetric detection system (Promega, Southampton, UK) was used according to the manufacturer's instructions with minor modifications concerning blocking and permeabilization of the whole-mount specimens with 2% Marvel and 0.5% Triton X-100 in PBS. The final product of peroxidase coupled to biotin was detected with amino-ethyl carbazole. A separate set of gut explants treated overnight with 4 mg of anisomycin (Sigma, Poole, Dorset, UK) was used as a positive control for apoptosis²² according to the manufacturer's suggestions.

Whole-Mount *In Situ* Hybridization and Immunohistochemistry

Prehybridization, hybridization, and posthybridization washes of whole embryos and dissected gastrointestinal tracts were performed as described before.^{14,23} Briefly, prehybridization, hybridization, and blocking with blocking reagent (Roche Diagnostics, Lewes, Beds, UK) were performed overnight. The probe for *c-ret*¹⁹ was used at concentrations of 25 to 100 ng/ml of hybridization buffer. Prehybridization, hybridization, and posthybridization washes were performed at 70°C. The probe was visualized using alkaline phosphatase-conjugated anti-digoxi-

genin antibody and BM Purple AP (both from Roche Diagnostics). The color reaction was stopped by washing overnight in PBS/10 mmol/L ethylenediaminetetraacetic acid (EDTA) and the signal was preserved by overnight incubation in 4% paraformaldehyde in PBS. Control experiments with probes omitted to check endogenous alkaline-phosphatase activity were performed at each developmental stage of the guts. Whole-mount embryos or gut primordia were photographed after *in situ* hybridization using a Zeiss Axiophot microscope with Nomarski optics (Jena, Germany). A rabbit polyclonal antibody against Hoxb5¹⁸ and monoclonal antibody against α -SMA (Sigma) in conjunction with *c-ret* *in situ* hybridization were used in this study to monitor enteric neuromuscular development. Detection was performed as previously described.¹⁸ Control experiments with primary antibody omitted were also performed.

Vibratome Sections

Some hybridized or immunostained whole-mount specimens were sectioned using a vibratome to determine the cellular distribution of the products in transverse or sagittal planes across the gut wall. Specimens were incubated in an embedding mix (gelatin, albumin, and sucrose) orientated and solidified with the addition of 25% glutaraldehyde. Sections were cut 30- to 50- μ m thick and mounted in Citifluor (Agar Scientific, Stansted, Essex, UK) and sealed with nail varnish for a photography using a Zeiss Axiophot microscope with Nomarski Optics.

Results

Overview

Maternal RA injection at E9.5 induced truncation of the posterior body axis together with tail bud agenesis in all 39 harvested embryos coexistent with stage-dependent morphological changes in gastrointestinal tracts. RA induced ectopic expression of *c-ret* and Hoxb5 in the central and peripheral nervous system. In the gastrointestinal tracts of RA-treated embryos, impaired cecal bud formation and delayed gut looping was observed in the first half of gestation associated with malrotation of the caudal ileum adjacent to the cecum in 31 embryos collected at the age of 10.5 to E12.5 dpc. These changes were associated with a delayed onset of α -SMA expression and migration of *c-ret*-expressing enteric neuronal precursors into the hindgut. In the rostral gut, RA induced ectopic expression of *c-ret* and Hoxb5 in the esophagus. Similarly, RA-treated E10.5 gut explants showed a lack of α -SMA immunoreactivity and delayed migration of *c-ret*-expressing neural crest cells into the postcecal intestine. These changes were coexistent with RA-induced ectopic *c-ret* and Hoxb5 expression in the esophagus in the explant. At E16.5 in five of eight embryos morphological caudal gut anomalies in the putative anorectal region were noted *in vivo*.

Figure 1. Morphological changes in affected embryos and gastrointestinal tract after *in vivo* RA treatment compared with controls. **A:** Control, vehicle-treated embryo (E10.5) with normally developed tail (**arrow**) and small proportion of apoptotic cells detected in the prevertebral region and at the caudal extremity of the tail bud. **B:** Degeneration of the embryonic tail because of apoptosis (dark red color) detected with TUNEL method in the caudal extremity of the tail bud in affected embryo at E10.5. **C** and **D:** Difference in the appearance of E12.5 control embryo (**C**) and embryo affected with RA-induced truncation of caudal body axis (**D**). **E:** Caudal extremity of a gut whole mount showing the anorectal region (**arrow**) and neural crest cell precursors expressing *c-ret* in control embryo. **F:** Sagittal vibratome section through the caudal extremity of the gut in the anorectal region shown with **arrow** in **D** showing normally developed rectum after immunostaining with Hoxb5. **Arrow** shows mesodermal layer surrounding gut epithelium. **G:** Ring-like constriction (**arrowhead**) in the caudal extremity of E16.5 gut whole mount after RA treatment in the corresponding anorectal level as shown in **E**. **H:** Sagittal vibratome section through the caudal gut extremity shown in **G**. **Arrowhead** shows ring-like constriction. **I:** Sagittal vibratome section through the ring-like malformation (**arrowhead**) obstructing the anorectal canal. **J:** Sagittal vibratome section through the blind-ending caudal gut extremity (**arrowhead**) at E16.5 after RA treatment. **K:** Appearance of the cecal bud (**asterisk**) at E11.5 in gut whole mount in control embryo. **L:** Poorly formed cecal bud (**asterisk**) at (E11.5) in RA-treated embryo. **M:** Well-developed cecal bud (**asterisk**) in gut whole mount at E12.5 in control, vehicle-treated embryo. **N:** Gut whole mount (E12.5) after RA treatment showing a poorly formed cecal bud (**asterisk**). **O** and **P:** Ventral view of gut whole mounts (E12.5); control (**O**) showing well-formed second gut loop, RA-treated (**P**) showing an absence of the second gut loop (**asterisk**) resulting in the malrotation of this gut region, which prevents the cecum to be exposed on the ventral site of the gut as seen in the control. Scale bars, 2 mm (**C** and **D**), 1 mm (**A**, **B**, **E**, **G**, and **H**), 0.5 mm (**F**, **K**, **L**, **O**, and **P**), and 0.2 mm (**M** and **N**). Abbreviation: e, epithelium.

Morphological Anomalies in Embryos and Guts Treated in Vivo with RA (Figure 1)

At E10.5, all 39 embryos harvested from RA-injected mice demonstrated a truncated posterior body axis. Apoptotic cells were seen in the degenerating tail and limb buds and in the whole prevertebral region as assessed by whole-mount TUNEL staining. In vehicle-treated embryos, by contrast, only a small proportion of apoptotic cells were present in the caudal extremity of well-developed tail bud (Figure 1, A and B). No apoptotic cells were detected, however, in the gut (data not shown). RA treatment resulted at E12.5 in a lack of tails and shorter caudal body regions characteristic of the caudal regression syndrome, as compared to control (Figure 1, C and D). In addition, at E16.5 there were anorectal ring-like constrictions, in five of the eight (62%) RA-treated embryos examined but these changes were not found in controls (Figure 1; E to J). Moreover in all 31 embryos with caudal agenesis (100%) there were morphological gut anomalies

lies at earlier stages of gut development. These included impaired outgrowth of the cecal bud observed in control E11.5 and E12.5 embryos (Figure 1; K to N) accompanied by delayed formation of the second intestinal loop at E12.5 (Figure 1O) and malrotation of the caudal ileum adjacent to the cecum (Figure 1P).

RA-Induced Impaired Development of Gut Neuromusculature in Vivo (Figures 2 and 3)

In controls at E10.5, α -SMA immunoreactivity was absent in the gut but was noted in the adjacent vitelline aorta (not shown). Significant α -SMA immunoreactivity was first noted at E11.5 in cells located at the ventral site of rostral, prececal gut. In contrast, RA-treated guts of the same age lacked α -SMA expression although attached vitelline aortas were positive (Figure 2, A and B). At E12.5 in controls α -SMA was expressed in the whole small intestine up to the cecal level (Figure 2C), whereas in RA-

Figure 2. Gut whole mounts and transverse vibratome sections after *in vivo* RA treatment showing α -SMA, Hoxb5 immunostaining, and *in situ* hybridization for c-ret compared with controls. **A:** Onset of α -SMA immunoreactivity in rostral, prececal gut in E11.5 control gut (**arrow**). The rest of the prececal gut shows the reflection of α -SMA-positive vitelline artery attached to the ventral side of the gut. **B:** Dorsal (**top**) and ventral (**bottom**) view of gut whole mounts (E11.5) after RA treatment showing absent α -SMA immunostaining (**arrow**) in the same region of the prececal gut as shown in **A**. The immunostaining seen in the prececal gut is the reflection of α -SMA-positive vitelline artery (**arrowhead**) attached to the dorsal side of the gut as shown at the **top**. **C:** Control gut whole mount (E12.5) showing α -SMA expression in the whole prececal gut up to the cecal level (**asterisk**). **D:** Gut whole mount (E12.5) after RA treatment showing delayed onset of α -SMA expression in the rostral, prececal gut (**arrow**). **E:** Sagittal vibratome section through the gut of control embryo (E12.5) showing well-developed circular muscle layer in the peripheral mesoderm of the small intestine (**arrow**). **F:** Sagittal vibratome section through RA-treated gut at E12.5 showing α -SMA-positive (**arrow**) poorly differentiated peripheral mesoderm in the rostral small intestine. **G:** Control gut whole mount (E11.5), *in situ* hybridization for c-ret showing neural crest-derived enteric neuronal precursors colonizing the whole prececal gut, the cecum (**asterisk**) and rostral, postcecal gut. **H:** *In situ* hybridization to c-ret-expressing neural crest cells present in the prececal gut up to the cecum level (**asterisk**) at E11.5 after RA treatment. **I:** Control gut whole mount (E12.5), *in situ* hybridization for c-ret in migrating neuronal precursors colonizing almost the whole large intestine (**arrow**). Caudal extremity of the large intestine shown by an **asterisk**. **J:** Gut whole mount (E12.5) after RA treatment, *in situ* hybridization for c-ret in neuronal precursors colonizing a large intestine adjacent to the cecum (**arrow**). Caudal extremity of the large intestine as in **I** shown by an **asterisk**. **K:** Control gut whole mount (E12.5) immunostained for Hoxb5 in the enteric neuronal precursors in the small intestine up to the cecal bud (**arrow**). **L:** Gut whole mount (E12.5) after RA treatment immunostained for Hoxb5 in the enteric neuronal precursors present in the small intestine up to the cecal bud (**arrow**). Stomach shown with an **asterisk**. Scale bars, 1 mm (**A–D**, **G**, **I–L**), 0.5 mm (**H**), 100 μ m (**E** and **F**).

treated guts of the same age, α -SMA expression was confined to the rostral small intestine, resembling the pattern observed 24 hours earlier (E11.5) in vehicle-treated controls (Figure 2D). Vibratome sections showed that the circular muscle layer expressing α -SMA remained poorly differentiated in RA-treated gut at E12.5 in comparison with the controls (Figure 2, E and F). At E16.5, α -SMA immunoreactivity was present in the whole gut of the controls and RA-treated embryos (not shown).

The expression of c-ret was examined at different stages as a marker for gut colonization by neural-crest derived enteric neural precursors. In E10.5 controls, c-ret-expressing cells were colonizing the prececal gut and at E11.5 expanded into the cecum and rostral postcecal gut (Figure 2G). After RA treatment by E11.5, c-ret-positive cells were only present in the prececal gut (Figure 2H). In E12.5 controls, c-ret-expressing cells colonized almost the whole large intestine (Figure 2I) whereas in RA-treated embryos c-ret-positive cells were noted in the cecum and adjacent rostral large intestine (Figure 2J). By E16.5, c-ret-expressing cells were detected in the whole intestine both in controls and RA-treated intestines (not shown).

In contrast to the migration pattern of c-ret-positive cells, Hoxb5 immunoreactivity in control and RA-treated

guts was noted only in the prececal gut and small intestine at E10.5, E11.5, and E12.5 (Figure 2, K and L). However, in rostral gut Hoxb5 expression pattern was different after RA treatment from that in controls. In controls, Hoxb5 rostral expression boundary was confined to the stomach in all developmental stages examined, whereas in RA-treated guts ectopic Hoxb5 expression was noted in the esophagus at E10.5 and E11.5 but not at E12.5 (Figure 2, K and L, and Figure 3, A and B). This observation was confirmed on vibratome sections (Figure 3, C and D). Similarly, in contrast to controls at E10.5 and E11.5, ectopic c-ret expression was found in the esophagus after RA treatment (Figure 3, E and F).

In addition, in whole control embryos at E10.5, the anterior Hoxb5 expression boundary in the hindbrain was between rhombomeres 7 and 8 at the level between somites 4 and 5 (Figure 3G). In RA-treated embryos, there was a rostral shift of expression up to the level of rhombomere 4 at the preotic hindbrain (Figure 3H). In control E10.5 embryos, c-ret expression was observed in the cranial ganglia, and also in dorsal root ganglia and the sympathetic chain of the peripheral nervous system (Figure 3I). After RA treatment, ectopic c-ret expression appeared in the midbrain, in the caudal branchial arches, and in the dorsal rami of the spinal ganglia (Figure 3J).

Figure 3. Hoxb5 immunohistochemistry and *in situ* hybridization for c-ret in whole mounts of embryo, gut, and vibratome sections after RA treatment compared with controls. **A:** Control gut whole mount (E11.5) immunostained for Hoxb5 showing rostral expression boundary in the stomach (asterisk) and caudal expression limit at the cecal bud level (arrow). **B:** Gut whole mount (E11.5) after RA treatment immunostained for Hoxb5 showing a rostral shift of the rostral expression boundary into the esophagus (asterisk). **C:** Transverse vibratome section of control gut whole mount (E11.5) at the level of the lung buds showing no Hoxb5 immunostaining in the esophagus (asterisk). **D:** Transverse vibratome section of a gut whole mount (E11.5) after RA treatment showing the presence of Hoxb5-positive cells in the esophagus (asterisk). **E:** Control gut whole mount (E11.5) hybridized for c-ret (blue) and immunostained for Hoxb5 (red) showing the presence of c-ret-positive cells in the vagus lateral to the esophagus (arrowhead). No c-ret-positive cells are seen in the esophagus. **F:** Gut whole mount (E11.5) hybridized for c-ret (blue) and immunostained for Hoxb5 (red) after RA treatments showing the presence of c-ret-positive cells in the esophagus (asterisk). **G:** Whole-mount control embryo (E10.5) showing the anterior expression boundary of Hoxb5 in the embryonal hindbrain (arrowhead). **H:** Whole-mount embryo (E10.5) after RA treatment showing a rostral shift of Hoxb5 expression to the preotic hindbrain (arrowhead). **I:** Whole mount of a control embryo (E10.5) with c-ret expression present in the cranial sensory ganglia (arrow). **J:** Whole mount of an embryo (E10.5) after RA treatment showing ectopic c-ret expression in the midbrain (arrowhead) and caudally in the dorsal rami of the spinal nerves (asterisk). Arrow indicates the otic vesicle located between rhombomeres 4 and 6 of the rostral and caudal hindbrain. Scale bars: 1 mm (A, E–J), 0.5 mm (B), 200 μ m in (C and D).

RA-Induced Morphological Changes in Gut Explants (Figure 4)

The results of the preliminary experiments showed that 10^{-5} mol/L of RA induced the appearance of unlooped

Figure 4. Whole-mount *in situ* hybridization for c-ret, immunostaining for Hoxb5 and α -SMA, and TUNEL method for apoptotic cells in gut explants after 72 hours in culture in controls and after RA treatment. **A:** α -SMA immunoreactivity in a cultured, control gut explant showing strong expression in the prececal gut (asterisk) close to the well-developed cecal bud (arrow). **B:** Gut explant treated overnight with RA showing an absence of α -SMA immunoreactivity in the prececal gut including the poorly developed cecum (arrow). **C:** Control gut explant after 72 hours in culture showing c-ret expression. Note the well-developed gut loops and the cecal bud (arrow). C-ret-expressing neural crest cells colonize the entire gut including postcecal region (asterisk). **D:** Dorsal side of RA-treated gut explant showing c-ret expression in the esophagus (arrowhead) and the prececal gut but not in the postcecal gut (asterisk). Note the poorly developed gut loops and the rudimentary cecum (arrow). **E:** Control gut explant showing strong Hoxb5 expression in the prececal gut (arrowhead). **F:** RA-treated gut explant showing a rostral shift of Hoxb5 expression to the esophagus (arrowhead). Cecum (arrow). **G:** TUNEL-stained RA-treated gut explant showing no apoptotic cells. Cecum (arrow). **H:** TUNEL-stained control gut explant showing the presence of apoptotic cells in the prececal gut up to the cecal bud (arrow) after apoptosis induced by anisomycin. Scale bars, 1 mm (A–H).

gut with an underdeveloped cecum in 10 of 10 (100%) of treated explants without stimulating apoptotic cell death, as compared to 10^{-4} mol/L of RA in which apoptosis was induced. RA (10^{-6} mol/L) produced morphological changes in 5 of 10 (50%) of explants whereas 10^{-7} mol/L of RA induced an altered gut appearance in 3 of 10 (30%) treated explants. No morphological changes or apoptotic cells were detected in control explants.

All 10 gut rudiments explanted at E10.5 and cultured in serum-free control conditions for 72 hours demonstrated the development of the cecal bud and prececal gut loops as occurs *in vivo* up to E12.5. Overnight RA treatment induced impaired growth of these structures, broadly similar to changes observed *in vivo* (Figure 4; A to F). These morphological changes coexisted with alterations

in gut neuromuscular development as assessed by α -SMA, c-ret, and Hoxb5 expression.

All 10 control explants examined showed strong α -SMA expression in the whole gut up to the start of the cecum (Figure 4A) that paralleled observations in the controls *in vivo* at E11.5 to 12.5. After RA treatment, all 10 explants of the same age showed a lack of α -SMA reactivity at the end of 72 hours of culture (Figure 4B).

In control explants, c-ret-expressing cells colonized the whole postcecal gut after 72 hours of culture in 9 of 10 explants (90%) whereas RA-treated explants showed impaired colonization of the postcecal intestine in 8 of 10 explants (80%) (Figure 4, C and D). In untreated gut explants the rostral limit of c-ret and Hoxb5 expression was in the dorsal stomach and the midgut (Figure 4, C and E). After RA treatment, Hoxb5 expression remained unchanged in the midgut but appeared rostrally in the esophagus in all explants examined (Figure 4F). Similar rostral expansion of c-ret expression into the esophagus was observed in all gut explants cultured in the presence of RA (Figure 4D). All these changes paralleled those noted *in vivo*. In the RA-treated gut explants a few apoptotic cells were noted. In contrast, control gut cultured overnight with anisomycin, a known inducer of apoptosis, showed the presence of many apoptotic cells (Figure 4, G and H).

Discussion

The retinoid-signaling system depends on a specific dietary component, vitamin A, from which the metabolically active retinoids are derived. Both clinical and experimental studies have shown that retinoids exert a wide variety of effects on vertebrate development concluding that both excess and deficiency of vitamin A are teratogenic to the developing fetuses.^{10,24–26} In the mouse embryo, RA-induced caudal agenesis is associated with apoptotic degeneration of the tail bud.⁷ In agreement with this observation, apoptotic cells were present in the degenerating embryonic tail after RA treatment, but not in the digestive tract both *in vivo* and *in vitro*. RA-induced apoptotic agenesis of the embryonic tail has been previously associated with specific down-regulation of Wnt-3a or Wnt5a expression that parallels the changes described in the homozygous mouse mutant for this gene.^{7,27,28} The lack of apoptotic cells in the gut primordia together with stage-specific gut anomalies suggest that RA-induced changes are not caused by RA toxicity but represent a specific effect.

The nature of the effects of RA on gut development is primarily unknown and in particular the responsiveness of gut neuromusculature to exogenous RA has not been previously studied. Gut morphogenesis involves numerous interacting processes that are spatially and temporally regulated and coordinated, which are concomitant with gut elongation and looping and with the outgrowth of asymmetrical structures such as the stomach and the cecum. These events coincide with the colonization of the gut by neural crest-derived enteric neuronal precursors. All these complex processes involve intense proliferation

and differentiation of gut layers because of inductive signals produced by gut endoderm and mesoderm.²⁹ The presence of endogenous RA in the developing gut is indicated by the expression of genes implicated in RA synthesis or metabolism that in the gut coexist with the expression of RAREs, RARs, and retinol-binding proteins.^{13,30–32} As teratogenic effects of RA are transduced by specific retinoid receptors³³ it would be conceivable to conclude that increased RA levels both *in vivo* and *in vitro* may interfere with natural morphogenetic processes including gut looping, rotating, and growth of asymmetrical structures such as the cecum resulting in the appearance of an underdeveloped cecum and second gut loop coexistent with malrotation of the caudal ileum adjacent to the cecum. The teratogenic effect of RA persisted in a proportion of older embryos that showed ring-like constrictions at the caudal gut extremity resulting in the blind-ending rectum. These RA-induced gut malformations included imperforate rectum, malrotation, or situs inversus associated with changes in the gut neuromusculature are also reported in patients with caudal regression syndrome or sirenomelia, VATER/VACTERL associations, or Currarino triads.^{34–42} A number of studies have previously demonstrated that either an excess or deficiency in the retinoid signal results in laterality defects in vertebrate embryos. A recognized anomaly of disturbed heart looping in mouse embryos is caused by teratogenic doses of RA, and the development of uncoiled intestine with delayed expression of intestinal α -SMA has been also described after stage-dependent RA treatment of *Xenopus* embryos.⁴³ The target genes of RA signaling in the gut are unclear but misexpression of homeobox transcription factor, *pitx2*, suggests that it could be a key factor in the process of heart and gut looping/rotation in *Xenopus*.⁴⁴ In the mouse, heart looping involves temporally and spatially restricted activity of transcription factors, extracellular matrix, and cytoskeleton proteins including actin.⁴⁴ These reports are in agreement with our data that showed that RA treatment affects gut looping/rotation and α -SMA expression both *in vivo* and *in vitro*. A previous study suggests that the initiation of enteric smooth muscle differentiation in the gut, can be induced by putative diffusible factors or their gradients produced by intestinal endoderm.²⁰ Interestingly, vascular α -SMA expression can be directly regulated by peptide growth factor, such as transforming growth factor- β (TGF β),⁴⁵ which is also expressed by the developing gut endoderm and is known to be involved in the induction of mesodermal differentiation in early murine embryos.^{29,46} Impaired gut looping and cecal development associated with malrotation of the caudal ileum and transiently perturbed determination and differentiation of enteric smooth muscle cells by RA cannot be explained in terms of overall developmental delay as the differentiation and morphogenesis of rostral structures such as eyes, ears, and frontonasal mass structures in the affected embryos appear to be normal when compared to control embryos. Similarly, RA-induced caudal regression is associated with morphological alterations in the more caudal gut whereas the esophagus and the stomach remained unchanged. Moreover, α -SMA expression in vitelline artery

associated with the gut appears to be the same as for control embryos and therefore can serve as an internal control of development and specificity of RA action in the gut. It can be concluded, that differentiation of enteric smooth muscle cells at the correct time could represent an essential event for the proper differentiation and morphogenesis of the digestive tract.

The interference of RA with cecal bud formation suggests a role for RA signaling in the regulation of putative genes involved in the morphogenesis of this gut region. It is of interest that nested *Hox* gene expression has been described in the cecum during murine development.^{14,47} Defects in homeobox genes are also found in patients with sacral agenesis⁴⁸ and in mutant mice where gene-targeted mutations of 5' *Hox* genes such as *Hoxd13* and *Hoxa13/Hoxd13* cause severe disorganization or lack of rectal musculature.^{49,50} In addition, disorganization or missing muscle layers in the muscularis propria and dysplasia of enteric nervous system have been described in patients with congenital anal atresia and rectal stenosis.^{15,16} These observations could be relevant to the presence of ring-like constrictions in the caudal gut found in a proportion of embryos after *in vivo* RA treatment. We found that RA-induced changes in the gut appearance *in vivo* paralleled those seen in *in vitro* experiments. The lack of α -SMA reactivity coexisted with delayed migration of *c-ret*-positive neural crest cells to colonize the postcecal gut indicates an important role of the differentiated mesoderm for the migration of enteric neuronal precursors. After RA treatment *in vivo* and *in vitro*, the appearance of the rostral gut was not changed, however, a stage-specific, rostral shift of the expression of *Hoxb5* and *c-ret*, markers of the developing enteric nervous system, was observed in the esophagus. These changes paralleled the rostral shift of the expression boundary of these genes observed in the embryonal hindbrain after *in vivo* RA treatment. Previous studies have suggested that homeobox gene expression, including *Hoxb5* can be regulated by RA *in vivo* and that RA induces their ectopic expression that often leads to specific morphological transformations.⁵¹ In the neural tube and vertebral column, *Hox* genes are expressed with distinct anterior boundaries set by RA-based signaling. They respond to the addition of exogenous RA by a rostral shift in their expression domains often associated with homeotic transformation of the hindbrain segments or vertebrae.^{5,52,53} The involvement of RA in the regulation of the expression of homeobox genes such as *Hoxa1* and *Hoxb1* in the endoderm of the anterior intestinal portal has been previously reported.^{54–56} It has been also shown that some *Hox* genes contain RA-responsive elements in their promoter regions.^{54–56} Exogenous RA also induced ectopic expression of *c-ret* in the embryonic spinal nerves that suggests RA involvement in the regulation of *c-ret* expression. This is in agreement with previous reports describing RA involvement in the regulation of *c-ret* expression in the cranial ganglia and in the neural tube in chicken embryos⁵⁷ and in the developing visceral organs such as the kidney.⁵⁸ Our results indicate that the distribution of cells expressing markers of neural crest cells in the esophagus is affected by RA treatment because it is

still unclear if the ectopic expression of *c-ret* and *Hoxb5* has been induced in cells that normally do not express these markers or is the result of altered migration of cells that usually express them.

The results presented in this study suggest that stage-specific *in vitro* and *in vivo* treatment of the developing gut with all-trans RA affects gut development by interfering with gut looping/rotation and with the growth of asymmetrical structures such as the cecum. These changes were coexistent with persistent teratogenic changes in the caudal gut extremities. Moreover, differentiation of the gut mesoderm and enteric nervous system as indicated by the expression of specific markers such as α -SMA, *c-ret*, and *Hoxb5* was also affected. These observations demonstrate that RA has a direct effect on gut morphogenesis and innervation and that the RA-induced caudal agenesis is a useful animal model to study gut responsiveness to exogenous teratogenic agents such as RA.

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