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Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth in vitro

Yoshiharu Sato, Robert O. Heuckeroth *

Department of Pediatrics, Department of Developmental Biology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8208, St. Louis MO 63110, USA

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ABSTRACT

Enteric nervous system (ENS) precursors undergo a complex process of cell migration, proliferation, and differentiation to form an integrated network of neurons and glia within the bowel wall. Although retinoids regulate ENS development, molecular and cellular mechanisms of retinoid effects on the ENS are not well understood. We hypothesized that retinoids might directly affect ENS precursor differentiation and proliferation, and tested that hypothesis using immunoselected fetal ENS precursors in primary culture. We now demonstrate that all retinoid receptors and many retinoid biosynthetic enzymes are present in the fetal bowel at about the time that migrating ENS precursors reach the distal bowel. We further demonstrate that retinoic acid (RA) enhances proliferation of subsets of ENS precursors in a time-dependent fashion and increases neuronal differentiation. Surprisingly, however, enteric neurons that develop in retinoid deficient media have dramatically longer neurites than those exposed to RA. This difference in neurite growth correlates with increased RhoA protein at the neurite tip, decreased Smurf1 (a protein that targets RhoA for degradation), and dramatically decreased *Smurf1* mRNA in response to RA. Collectively these data demonstrate diverse effects of RA on ENS precursor development and suggest that altered fetal retinoid availability or metabolism could contribute to intestinal motility disorders.

Introduction

The enteric nervous system (ENS) controls most aspects of intestinal function including intestinal motility, blood flow, and epithelial secretion. The ENS also responds to sensory stimuli from the bowel wall and gut lumen. To perform these functions there are estimated to be 500 million enteric neurons in 20 functional classes in humans (Furness, 2006). All of these neurons, and their supporting glia are derived from a small group of neural crest cells that originate in the vagal, sacral and upper thoracic neural tube (Gariepy, 2001; Heanue and Pachnis, 2007; Newgreen and Young, 2002a,b). These ENS precursors migrate through the fetal bowel wall, undergo sequential lineage restriction and then differentiate, but mechanisms controlling ENS precursor migration, proliferation, and differentiation remain incompletely understood. When these crest-derived precursors fail to reach the distal bowel, the aganglionic bowel tonically contracts resulting in life threatening intestinal obstruction that in humans is called Hirschsprung disease, an illness affecting 1:5000 infants (Skinner, 1996). A variety of less well understood human motility disorders called intestinal pseudo-obstruction syndromes may also result from both damage to the ENS and abnormal ENS development. For this reason, defining the molecular mechanisms of ENS precursor differentiation may provide novel methods to prevent intestinal motility disorders.

Two recent studies suggested that the active vitamin A metabolite retinoic acid may have important roles in ENS development. The first study demonstrated that excess retinoic acid during fetal development results in delayed colonization of the distal bowel by ENS precursors (Pitera et al., 2001). Retinoic acid toxicity also caused abnormal gut mesenchyme differentiation, and other abnormalities of gut looping, rotation and morphogenesis. The second study demonstrated aganglionosis of the bowel in retinaldehyde dehydrogenase 2 (*Raldh2/Aldh1a2*) deficient mice that had been partially "rescued" by treatment of the pregnant mothers with all-*trans*-retinoic acid (RA) (Niederreither et al., 2003). Because Raldh2 protein is critical for local production of RA in many regions of the developing embryo, *Raldh2*$^{-/-}$ mice remain quite abnormal even after RA supplementation including serious defects in gut mesenchyme and in other neural crest derivatives. Thus, these studies suggest that RA influences ENS precursor development, but do not demonstrate direct effects of RA on ENS precursors or define how RA signaling influences the proliferation, survival or differentiation of these cells. In particular, it is possible that the observed effects in vivo result from RA induced changes in the microenvironment through which ENS precursors migrate. In *Raldh2*$^{-/-}$ mice, it is also possible that the distal bowel aganglionosis results from defects in neural crest development that occur long before ENS precursors reach the bowel.

* Corresponding author. Fax: +1 314 286 2893.
E-mail address: Heuckeroth@kids.wustl.edu (R.O. Heuckeroth).

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To determine if RA has any direct effects on ENS precursors, we have used immunoselection to separate crest-derived cells in the fetal gut from other cells within the gut wall and then maintained these ENS precursors in low density culture with or without added RA. These studies demonstrate significant effects on ENS precursor proliferation, neuronal differentiation, and neurite extension in vitro that suggest that RA influences many aspects of ENS development via direct effects on ENS precursors that are independent of retinoid induced changes in gut mesenchyme differentiation or RA effects on earlier stages of neural crest development. Furthermore, our studies demonstrated unexpectedly that while RA increases neuronal differentiation, it also reduces neurite growth from ENS precursors. This reduction in neurite growth correlates with a RA induced reduction in Smurf1 protein and mRNA abundance and an increase in RhoA protein levels in developing enteric neurons. Collectively these data demonstrate complex effects on many aspects of ENS development suggesting that vitamin A deficiency or excess could cause defective ENS development and contribute to human motility disorders.

Methods

Primary culture of non-selected and immunoselected ENS precursors

E12.5 CF-1 mouse stomach, small bowel, and colon were dissociated with collagenase (0.5 mg/mL) and dispase (0.5 mg/mL) to yield a single cell suspension. Cells were filtered through a 40 μm cell strainer (BD Falcon). Non-selected dissociated gut cells were plated at 10,000 cells/well in poly-D-lysine/laminin coated 8-well chamber slides (Biocoat, Fisher) and grown in Neurobasal medium plus vitamin A deficient B27 supplement (Invitrogen), glial cell line-derived neurotrophic factor (GDNF, 50 ng/mL) and 2 mM L-glutamine (Invitrogen). To immunoselect enteric neural crest-derived cells, dissociated gut cells were exposed to $p75^{NTR}$ antibody (Chemicon, 1:1000) in vitamin A deficient B27 supplemented Neurobasal medium followed by incubation with goat anti-rabbit coupled paramagnetic beads (Miltenyi Biotec GmbH, 1:50, 1 h, 4 °C) before separating neural crest-derived cells from unselected cells with a positive selection column (MACS Separation columns, Miltenyi Biotec GmbH). Immunoselected cells were plated at 1000 cells/well using the same conditions as unselected cells. This low cell density was used to minimize cell–cell interaction and to maintain approximately the same number of crest-derived cells as in the non-selected cultures. In all the experiments, factors tested were added to the medium at the time of plating. Experimental sets were as follows: (a) defined RA-free medium plus 10^{-6} M all-*trans* retinoic acid (RA) (Sigma), (b) defined RA-free medium only, or (c) defined RA-free medium plus 10^{-5} M pan-retinoic acid receptor inhibitor (BMS493, generously provided by Dr. Chris Zusi at Bristol-Myers Squibb). RA and BMS493 were dissolved in ethanol to prepare stock solutions. One μL of each stock solution was added to 10 mL of medium. Control cultures also had 1 μL of ethanol per 10 mL of medium. Cells were maintained in a humidified environment with 5% CO_2 at 37 °C for 2 days or 7 days. For prolonged culture, medium was changed every 2 days by withdrawing half of the medium and adding new medium. For proliferation analysis, bromodeoxyuridine (BrdU, 10 μmol/L final concentration) was added to cells 5 h before fixation. Cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (room temperature, 25 min) for immunohistochemical analysis. All experiments were performed in triplicate.

Immunohistochemistry and image analysis

After fixation, cells were washed with PBS, blocked with 4% normal donkey serum in TBST (Tris-buffered saline plus 0.1% Triton X-100) (1 h, 37 °C) and then incubated in primary antibody (4 °C overnight). Primary antibodies include: goat anti-Ret (1:100; Neuromics), rabbit TuJ1 (1:500) and mouse TuJ1 (1:100; Covance), rabbit anti-S100β (1:500; Dako), anti-RhoA (sc-179, 1:50), anti-Smurf1 (sc-25510, 1:50), anti-PI 3-kinase p85α (B-9) (sc-1637, 1:50), anti-Cdc42 (P1) (sc-87, 1:50; Santa Cruz Biotechnology), rabbit anti-phospho-PKCζ (p-PKCζ), anti-phospho-GSK3β (p-GSK3β) (1:100; Cell Signaling Technology) and rabbit anti-Raldh2 antibody (kind gift of Dr. Peter McCaffery, University of Aberdeen) (Berggren et al., 1999; Moss et al., 1998). Antibody binding was visualized with Alexa Fluor 350-, 488-, 546- and 594-conjugated anti-mouse, anti-goat and anti-rabbit secondary antibodies (1:250; 25 °C, 1 h, Invitrogen). For BrdU labeling, cells were treated with 4 N HCl (25 °C, 10 min) either before primary antibody (Ret/TuJ1/BrdU labeling) or after secondary antibody (S100β/BrdU labeling). BrdU labeling was then visualized after PBS washing, by incubation with Alexa Fluor 488-conjugated anti-BrdU antibody (1:20, 37 °C, 45 min, Invitrogen). Apoptotic cells were analyzed using an in situ Cell Death Detection kit (Fluorescein) (Roche) following the manufacturer's protocol. Images were obtained with an Olympus BX60 microscope, an Axiocam digital camera and AxioVision imaging software (Zeiss) using identical exposure times. Neurite length was measured by using NIH ImageJ 1.36. Cell counts were obtained manually by using a counting grid and a 20× objective.

In situ hybridization

Wild type CF-1 mice were perfused with cold 4% paraformaldehyde (PFA), post-fixed overnight at 4 °C and then frozen in OCT before sectioning at 14 μm thickness. Slides were warmed to 25 °C, baked 15 min at 50 °C and then fixed again in 4% PFA for 20 min at 25 °C. After washing twice in diethylpyrocarbonate treated phosphate-buffered saline (PBS-DEPC, 10 mM) for 5 min, tissues were digested in Proteinase K (25 μg/mL for E14 samples and 10 μg/mL for E12 samples) for 8–13 min in (50 mM Tris (pH 7.5), 5 mM EDTA, DEPC treated water). Slides were then washed again in PBS-DEPC (2×5 min), incubated in 4% PFA for (15 min, 25 °C), and rinsed in DEPC treated water. Tissues were then blocked with 0.2% acetic anhydride/0.1 M triethanolamine (10 min, 25 °C), washed in PBS-DEPC (5 min, 25 °C), and pre-hybridized for 1 h at 65 °C in pre-hybridization solution (50% formamide, 5× SSC, 1 mg/mL Yeast tRNA, 100 mg/mL Heparin, 1× Denhardt's Solution, 0.1% Tween 20 (Sigma P-1379), 0.1% CHAPS (Sigma C-3023), 5 mM EDTA pH 8.0). Riboprobes (2 ng/mL final concentration, prepared using a MAXIscript kit from Ambion) were then added to fresh pre-hybridization solution, slides were covered with coverslips, and tissues were hybridized overnight at 65 °C in humidified chamber. Following hybridization, tissues were washed in 1× SSC/50% formamide at 65 °C (3×30 min), then twice in PBT (10 mM PBS with 0.1% Triton X-100 and 2 mg/mL BSA) for 20 min at 25 °C, and then blocked with PBT/20% NSS (normal sheep serum) for 1 h at 25 °C. Hybridized probe was detected after incubation (overnight, 4 °C) with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche, 1:2000) in fresh blocking solution (PBT/20% NSS). During the detection step for some E12 samples, we also used the TSA Plus DNP System (Perkin Elmer) according to the manufacturer's instructions. Slides were then washed in PBT (3×30 min, 25 °C) followed by washing once in alkaline phosphatase (AP) buffer (100 mM Tris pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% TWEEN 20) with levamisole (5 mM, DakoCytomation) for 5 min and once in AP buffer without levamisole. Finally slides were incubated in AP buffer with 3.5 μL/mL BCIP (0.35% final concentration) and 1.5 μL/mL NBT (0.15% final concentration) for 1–3 days in dark at 4 °C, or until desired stain is attained. Plasmids used to generate probes for *Rarb* (exons 3–9), *Rxrb* (exons 1–10), *Rxrg* (exons 1–10) and *Cyp26c1* (exons 4–5) were generously provided by Dr. Pierre Chambon (IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), INSERM (National Institute of Health and Medical Research), CNRS (Centre National de la Recherche Scientifique)). Plasmids for *Raldh2* and *Raldh3* were generously provided by Dr. Ursula Drager (University of Massachusetts). For the other genes, cDNA was cloned via PCR and used as a template for riboprobe preparation. Primers used to isolate these cDNA are listed as follows: *Rara* (exons 7–9), 5′-TCTCCCTGGACATTGACCTC and 5′-ATGCTCCGAAGGTCTGTGAT; *Rarg* (exons 4–9), 5′-CTCGGGTCTATAAGCCATGC and 5′-CATAGCCAGCATTGTGCATC; *Rxra* (exons 4–9), 5′-GCTCACCAAATGACCCTGTT and 5′-GAAGAACCAGGTTGCTCCAG; *Raldh1* (exons 1–2), 5′-CATGCAAGGGTGCCTTTATT and 5′-CTGTCCCTCAGTGACTCCTTG; *Cyp26a1* (exons 1–4), 5′-CTGGGCGGCCTTATAAAGAG and 5′-CGAAATGTTCTCCTCGATGC; *Cyp26b1* (exons 4–5), 5′-TCAATGTGCCCAAGATCCTA and 5′-AGCCTTCTGTAGGCCCTTCT. Sense and anti-sense riboprobes were prepared for all in situ hybridization studies and no signal was detected for any of the sense controls (Supplemental Fig. 1).

β-Galactosidase histochemistry

For whole mount staining, tissues were fixed for 30 min on ice with 0.2% glutaraldehyde in 100 mM phosphate buffer (pH 7.4) (PBS) plus 2 mM $MgCl_2$, before washing twice (5 min each) in PBS plus 2 mM $MgCl_2$. The blue precipitate was generated by incubation in the dark at 37 °C in X-gal buffer (100 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P40, 5 mM potassium-ferricyanide, 5 mM potassium-ferrocyanide, and 1 mg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)). For sections, stained whole gut samples were frozen in OCT and 14 μm sections were cut with a cryostat.

Polymerase chain reaction (PCR) based analysis of gene expression

The following primers were designed to generate short amplicons (50–100 bp, Tm about 60 °C) and were synthesized by Integrated DNA Technologies Inc.:

RhoA: 5′-GAATGACGAGCACACGAGAC-3′ and 5′-GTACCCAAAAGCGCCAATCC-3′.
Smurf1: 5′-CACTGGCTACCAGCGTTTG3′ and 5′-CCTATTCTGTCTCGGGTCTGTAA-3′.
Rara: 5′-TTCTTTCCCCCTATGCTGGGT-3′ and 5′-GGGAGGGCTGGGTACTATCTC-3′.
Rarb: 5′-TCCTGGGAGTTGGTGATGTC-3′ and 5′-TCGGAGCAGCTCACTTCCTA-3′.
Rarg: 5′-ATGTACGACTGCATGGAATCG-3′ and 5′-CCAGTGGCTCTGCGTAGTAA-3′.
Rxra: 5′-ATGGACACCAAACATTTCCTGC-3′ and 5′-CCAGTGGAGAGCCGATTCC-3′.
Rxrb: 5′-GCAGCCCAAATGACCCAGT-3′ and 5′-GGAGAGGGACCGATCAAAGAT-3′.
Rxrg: 5′-CATGAGCCCTTCAGTAGCCTT-3′ and 5′-CGGAGAGCCAAGAGCATTGAG-3′.
Raldh1: 5′-ATACTTGTCGGATTTAGGAGGCT-3′ and 5′-GGGCCTATCTTCCAAATGAACA-3′.
Raldh2: 5′-CAGAGAGTGGGAGAGTGTTCC-3′ and 5′-CACACAGAACCAAGAGAGAAGG-3′.
Raldh3: 5′-GGGTCACACTGGAGCTAGGA-3′ and 5′-CTGGCCTCTTCTTGGCGAA-3′.
Cyp26a1: 5′-AAGCTCTGGGACCTGTACTGT-3′ and 5′-CTCCGCTGAAGCACCATCT-3′.
Cyp26b1: 5′-TCATCGGAGAGACTGGTCACT-3′ and 5′-GGTGCTCACTAGCTGGTGTTC-3′.
Cyp26c1: 5′-GCGTAGTCAAGGAGGTGCTG-3′ and 5′-AGCCTTTGGGGATCTGGTAA-3′.