Chronically administered retinoic acid has trophic effects in the rat small intestine and promotes adaptation in a resection model of short bowel syndrome

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Wang L, Tang Y, Rubin DC, Levin MS. Chronically administered retinoic acid has trophic effects in the rat small intestine and promotes adaptation in a resection model of short bowel syndrome. Am J Physiol Gastrointest Liver Physiol 292: G1559–G1569, 2007. First published February 15, 2007; doi:10.1152/ajpgi.00567.2006.— Following the loss of functional small bowel surface area, the intestine undergoes a compensatory adaptive response. The observation that adaptation is inhibited in vitamin A-deficient rats following submucosal intestinal resection suggested that vitamin A is required for this response and raised the possibility that exogenous vitamin A could augment adaptation. Therefore, to directly assess whether chronically administered retinoic acid could stimulate gut adaptation in a model of short bowel syndrome and to address the mechanisms of any such effects, Sprague-Dawley rats were implanted with controlled release retinoic acid or control pellets and then subjected to mid-small bowel or sham resections. At 2 wk postoperation, changes in gut morphology, crypt cell proliferation and apoptosis, enterocyte migration, the extracellular matrix, and gene expression were assessed. Retinoic acid had significant trophic effects in resected and sham-resected rats. Retinoic acid markedly inhibited apoptosis and stimulated crypt cell proliferation and enterocyte migration postresection. Data presented indicate that these proadaptive effects of retinoic acid may be mediated via changes in the extracellular matrix (e.g., by increasing collagen IV synthesis, decreasing E-cadherin expression, and reducing integrin β1 levels), via affects on Hedgehog signaling (e.g., by reducing expression of the Hedgehog receptors Ptc and Ptc2 and the Gli1 transcription factor), by increasing expression of Reg1 and Pap1, and by modulation of retinoid and peroxisome proliferator-activated receptor signaling pathways. These studies are the first to demonstrate that retinoic acid can significantly enhance intestinal adaptation and suggest it may be beneficial in patients with short bowel syndrome.

vitamin A; apoptosis; enterocyte migration; Hedgehog signaling; extracellular matrix

PATIENTS WITH INTESTINAL FAILURE or short bowel syndrome resulting from intestinal disorders such as Crohn’s disease, acute bowel infarction, and trauma are often dependent on parenteral nutrition. The remnant intestine has a limited capacity to adapt by increasing intestinal functional surface area. Interventions that promote adaptation are sought to reduce or eliminate dependence on parenteral nutrition and its associated costs and comorbidities. In rodent resection models, the adaptive response is characterized by crypt deepening and villus lengthening resulting from increases in crypt cell proliferation, enterocyte migration and differentiation, and changes in cell apoptosis. These resection models have demonstrated that adaptation is stimulated by luminal nutrients (e.g., see Ref. 6) and have identified peptides such as glucagon-like peptide 2 and growth hormone as putative promoters of the adaptive response (reviewed in Refs. 2, 4, 10, 28, 54). The limited clinical effectiveness and high costs of these trophic factors necessitate further investigations to better define the mechanisms underlying adaptation as a prerequisite for identifying proadaptive nutrients and more effective proadaptive growth factors.

In prior studies in rats subjected to submucosal small bowel resection, our group (9) observed that the expression of cellular retinol binding protein 2 and several vitamin A-responsive genes were upregulated in the remnant intestine at very early times postresection. This suggested that vitamin A may be required for adaptation. This hypothesis was supported by our demonstration that vitamin A deficiency inhibited adaptation at 48 h and 10 days postresection (49, 50), concurrent with increased crypt cell apoptosis and reduced crypt cell proliferation and enterocyte migration rates (50). Our group also showed that a single injection of all-trans retinoic acid (RA) could stimulate crypt cell proliferation in the remnant intestine in the first 6 h following partial small bowel resection (58).

These observations suggest that vitamin A is a putative regulator of the adaptive response and that exogenous vitamin A could augment adaptation (49, 50, 58). However, the potential for vitamin A to have a sustained effect on the intestinal mucosa, as required for effective therapies for short bowel syndrome, has not been demonstrated thus far. Therefore, the current studies were undertaken to directly assess the therapeutic potential of chronically administered RA to stimulate gut adaptation and to address the mechanisms of any such effects.

MATERIALS AND METHODS

Animals, experimental design, and surgery. All animal protocols were approved by the Washington University School of Medicine Animal Studies Committee. Adult male Sprague-Dawley rats (200–250 g) were obtained from Sasco (Omaha, NE) and were acclimated in the animal care facility for at least 72 h before surgery. Rats were provided a standard rat chow diet and water ad libitum and were maintained on a 12:12-h light-dark cycle. Rats were randomly assigned to experimental groups, using a 2 factorial design (i.e., sham resected or resected × RA or vehicle). This study design allowed the effects of RA (vs. placebo) and partial resection (vs. transection and reanastomosis) to be independently assessed.

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All-trans RA was administered using continuous time release pellets (20 mg, 21-day release; Innovative Research of America, Sarasota, FL). Matching placebo (vehicle) pellets contained all the matrix components of the RA pellets. To achieve elevated serum RA levels by the time of surgery, we implanted RA or placebo pellets subcutaneously in the lateral cervical region 2 days preoperatively.

The surgical protocol was described previously (50, 58). In brief, after overnight fasting, rats were anesthetized with inhalational halothane, pentobarbital sodium (40 mg/kg ip), and atropine (0.4 mg/kg im). Rats paired by weight underwent 70% small intestinal resection or sham resection. The remnant small intestine post-70% resection (i.e., the proximal 5 cm of the jejunum and the distal 15 cm of the ileum) was reanastomosed end to end using interrupted 6-0 silk sutures. For sham-resected rats the jejunum was transected 5 cm distal to the ligament of Treitz and reanastomosed end to end. Postoperatively, rats received intraperitoneal gentamicin (4 mg in 6 ml of normal saline) and a liquid diet containing sucrose (50 g/l) and oxytetracycline (4.5 g/l) for 24 h, followed by a standard chow diet. Rats were killed with pentobarbital sodium (150 mg/kg ip) at 2 wk postoperation. The small intestine was rapidly harvested and divided into duodenal-jejunal and ileal segments (i.e., proximal and distal to the anastomosis, respectively). After being flushed in cold phosphate-buffered saline (PBS), the intestinal lumen was opened longitudinally and the mucosa was scraped from the underlying muscle with a glass slide for isolation of protein and RNA. One full thickness portion of each segment was fixed in formalin and rolled along its transverse axis for histological analysis.

**Determination of serum and intestinal RA levels.** Mucosal samples (2–3 g) were homogenized with 3–5 ml of isopropanol-dichloromethane (2:1, vol/vol) spiked with 40 ng of retinyl acetate as the internal standard. The homogenates were transferred to a glass vial and stored on ice, followed by centrifugation. Sera were stored at −80°C. Serum RA was measured by a competitive enzyme-linked immunosorbent assay (ELISA) method as described (24, 25). The homogenates were filtered on the third day. The residual intestine (2–3 g) were homogenized with 3–5 ml of isopropanol-dichloromethane (2:1, vol/vol). Serum was prepared by incubating whole blood for 10 min on ice, followed by centrifugation. Sera were stored at −70°C until retinoids were extracted as described (3). Ethanol (0.1 ml, spiked with 20 ng of retinyl acetate as the internal standard), 0.2 ml of ethyl acetate, and 0.01 ml of acetic acid (10%, vol/vol) were added to 0.05 ml of serum aliquots. After vortexing and centrifugation, supernatants were transferred to new tubes and kept cold. The pellets were resuspended in hexane (0.1 ml) and centrifuged, and the supernatant was pooled with the initial supernatant. Water (0.2 ml) was added to the pool, and after vortexing and centrifugation, the upper organic phase was removed and evaporated under a gentle stream of nitrogen. The residue was dissolved in methanol-dichloromethane (3:1, vol/vol). Aliquots of the mucosal and serum extractions were separated by reverse-phase HPLC on a C18 column (Microsorb-MV 0.46 × 10 cm, 3-µm particle, 100-Å pore; Varian, Palo Alto, CA) using a 30-min linear gradient with methanol:water (3:1, vol/vol, containing 10 mM ammonium acetate, solvent A) and methanol:dichloromethane (1:1, vol/vol, solvent B) at a flow rate of 0.8 ml/min. Separations were monitored at 350 nm and quantitated by comparing the integrated peak areas to standard curves determined with purified standards.

**Morphometric analysis.** Villus heights and crypt depths were measured in 20–50 well-oriented hematoxylin-eosin-stained crypt-villus units with the aid of a slide micrometer and Scion Image software (beta 4.02; Scion, Frederick, MD) as previously described (44, 49, 50, 60). Small intestinal surface areas were calculated from morphological measurements as described previously (23).

**Analysis of apoptosis.** Apoptotic cells were identified by standard morphological changes, including nuclear condensation, perinuclear clearing, and cell shrinkage, and by staining for activated caspase-3. Briefly, tissue sections were incubated with citrate buffer (pH 6.0) in a pressure cooker (Biocare, Walnut Creek, CA) for 3 min at 15 lb./in.² to enhance antigen recognition. Slides were treated sequentially with avidin/biotin blocking (Vector Labs, Burlingame, CA), and protein block (Dako, Carpenteria, CA) and then incubated overnight at 4°C with rabbit anti-caspase-3 polyclonal antibody (1:50, Cell Signalling Technology, Beverly, MA). After incubations with goat anti-rabbit biotinylated IgG (1:800; NEN Life Science, Boston, MA) for 1 h at room temperature, endogenous peroxidase was quenched for 30 min in 1% hydrogen peroxide and streptavidin-horseradish peroxidase (SA-HRP; 1:1,000, Dako) was added for 1 h. Sections were detected with diaminobenzidine (DAB; Sigma, St. Louis, MO) and counterstained with hematoxylin. The number of apoptotic cells in 20–50 well-oriented longitudinal crypts was used to compute the apoptotic index (number of apoptotic cells per 100 crypt cells).

**Crypt cell proliferation.** Ninety minutes before being euthanized, rats were injected with 5-bromo-2′-deoxyuridine (BrdU; 120 mg/kg body wt; Sigma) and 5-fluoro-2′-deoxyuridine (12 mg/kg; Sigma) to label S-phase cells. For epitope recovery, tissue sections were incubated for 3 min in room temperature ReVeal buffer solution (Biocare Medical, Walnut Creek, CA) at 15 lb./in.². 5-BrdU was detected with a mouse anti-BrdU antibody (1:300 dilution; Biogenex, San Ramon, CA). After incubation with goat anti-mouse IgG (1:1,000; NEN Life Science) and SA-HRP (1:1,000; Dako), antigen-antibody complexes were detected with DAB. The proliferation index is the number of labeled cells per 1,000 crypt cells in 20–50 well-oriented, longitudinal crypts per section for each rat.

**Enteroctye migration.** Enteroctye migration was determined with our previously reported double labeling method (52). Briefly, 5-BrdU solution (5-bromo-2′-deoxyuridine:5-fluorodeoxyuridine, 10:1; Sigma) was injected intraperitoneally 49.5 h (4.8 mg/kg) and 1.5 h (120 mg/kg) before euthanasia. Cells that incorporated BrdU were detected as described above. The distance between the leading edge of BrdU-labeled enterocytes on the crypt and villus was used to calculate the migration rates.

**Immunohistochemical staining of laminin, fibronectin, and cadherin.** Paraffin-embedded tissue sections were incubated with rabbit anti-laminin (1:200; Sigma), rabbit anti-fibronectin (1:200; Sigma), or mouse anti-pan-cadherin (1:250; Sigma) for 1 h. The samples were then incubated with goat anti-rabbit biotinylated IgG (1:2,000; NEN Life Science) for laminin and fibronectin or goat anti-mouse biotinylated IgG (1:1,000; NEN Life Science) for cadherin. SA-HRP (1:1,000; Dako) was added for 30 min. The samples underwent tyramide enhancement (NEN Life Science) for 10 min, followed by reincubation with SA-HRP (1:1,000) for 30 min. The slides were developed with DAB.

**Immunofluorescence staining of collagen IV.** Tissue sections were incubated with goat anti-collagen IV (1:40; Chemicon, Temecula, CA) for 1 h and anti-goat rhodamine red (1:200; Jackson Immunoresearch, West Grove, PA) was then added for 30 min. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole, mounted with fluorescent mounting media (Dako, Golstrup, Denmark), and examined with a fluorescent microscope, and the density of collagen IV staining was quantified using Scion Image software (beta 4.02; Scion). At least 20 fields containing villi or and another 20 fields including crypts were photographed (×400). The density of red immunofluorescence staining was measured, and the density of collagen IV staining was calculated as mean density per field (50).
all of the primer sets that were used. For relative comparative quantitative PCR, the amount of target gene ($\Delta C_T$) was normalized by subtracting the critical threshold ($C_T$) of the endogenous control (18S rRNA) from that of the target gene ($\Delta C_T$). For intergroup comparisons, the relative change of the target gene equals $2^{-\Delta C_T}$, where $\Delta C_T = C_T - C_T$ of the tested sample - $C_T$ of the control sample.

**Statistical analysis.** The data are expressed as means ± SE. Single-factor and two-way ANOVAs were performed using SigmaStat (SigmaStat 2.03; SPSS, Chicago, IL). Differences with a P value <0.05 were considered to be significant.

**RESULTS**

Serum and small intestinal RA levels. RA pellets implanted 48 h preoperatively increased mean serum levels ~30% by the time of surgery (vehicle, 11.1 ng/ml; RA, 14.1 ng/ml; $P < 0.05$; Fig. 1). These differences were sustained at 2 wk post-operation (vehicle, 11.1 ng/ml; RA, 13.4 ng/ml; $P < 0.05$). In the small intestinal mucosa, RA (27.2 ng/g) and 9-cis-RA (18.4 ng/g) were only detected in the RA-treated rats (Fig. 2). As expected, retinyl esters accounted for the majority of intestinal retinoids in both treatment groups.

Expression of retinoid and peroxisome proliferator nuclear receptors. RA and its 9-cis isomer are endogenous ligands for nuclear RA (RAR, 9-cis RA) and rexinoid receptors (RXR, 9-cis RA). These receptors mediate many of the effects of retinoids directly or indirectly via the formation of heterodimeric complexes with each other or with other nuclear receptors [e.g., peroxisome proliferator activated receptors (PPAR)]. Thus, to further dissect the mechanism for the pro-adaptive effects of RA, the expression of nuclear retinoid and peroxisome PPAR were investigated. As shown in Table 2, RARB, RARY, RXRo, and RXRy mRNAs were significantly decreased in the ileal mucosa by resection in both vehicle- and RA-treated animals. Administration of RA alone had no effect. Expression of RXRo and RXRb were not influenced by
resection or RA treatment. PPARα was also decreased by resection only, whereas neither resection nor RA affected expression of PPARβ/δ and PPARγ.

**Morphological analysis.** A typical postresection adaptive response, characterized by significant crypt deepening and villus lengthening, occurred in the residual small intestine of RA and vehicle treated rats (Fig. 3). In the duodenal-jejunal segments, compared with paired transected controls, crypt depths were increased 1.16-fold in vehicle and 1.18-fold in RA-treated rats ($P < 0.05$), and villus lengths were increased 1.16- and 1.20-fold, respectively ($P < 0.05$). Changes were more pronounced in the ileum, where crypt depth increased 1.22- and 1.32-fold ($P < 0.05$) and villus lengths increased 1.40- and 1.46-fold ($P < 0.05$) in vehicle- or RA-treated rats, respectively.

Independent of surgery, RA significantly enhanced the adaptive response in resected rats and had significant trophic effects in the small intestine of transected rats. RA induced changes in crypt depths and villus lengths that resulted in significant increases in small intestinal absorptive area in the duodenal-jejunal and ileal segments (1.30- to 1.50-fold) of resected and sham-resected rats (see Fig. 3, E–G). Although surgery and RA independently enhanced intestinal surface area, their effects were not interactive [$P < 0.05$ for main effects, interaction was nonsignificant (NS) by 2-way ANOVA]. The changes in crypt depths and villus lengths were not due to changes in cell size but rather to hyperplasia (data not shown).

**Crypt cell proliferation.** To address the mechanisms for the intestinotrophic effects of RA, we first examined rates of crypt cell proliferation and apoptosis. Crypt cell proliferation was significantly and independently stimulated by resection and RA treatment in the duodenal-jejunal and ileal remnants [Fig. 4; for both segments: $P < 0.001$ for surgery effect, $P < 0.004$ for RA effect; $P > 0.05$ for surgery × RA interaction (NS)]. The relative changes with surgery or RA were consistent with the
was associated with significantly reduced ileal expression of RA-induced inhibition of apoptosis 61% in the ileal segment of transected rats and 47% in both segments; Fig. 5). The magnitude of the decrease in apoptotic index was ~52% in the duodenal-jejunal segment and 61% in the ileal segment of transected rats and 47% in both segments of resected rats. RA-induced inhibition of apoptosis was associated with significantly reduced ileal expression of Bax and caspase-3 mRNA levels (1.8-fold lower; Table 2). The reduction in Bax expression is consistent with data indicating that Bax is required for the resection-mediated enhanced apoptosis that is observed in some resection models (26, 47, 52, 53) The effects of surgery and RA treatment on apoptosis were not interactive.

**Table 2. Relative changes in ileal mRNA levels after partial small intestinal resection or RA treatment**

<table>
<thead>
<tr>
<th>Enterocytic function</th>
<th>Sham Resected (RA vs. Veh)</th>
<th>Resected (RA vs. Veh)</th>
<th>Vehicle (RE vs. SHR)</th>
<th>Retinoic Acid (RE vs. SHR)</th>
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<tr>
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<td>NS 1.7</td>
<td>1.4</td>
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<td>ApoA-IV</td>
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<td>–2.3 1.6</td>
<td>2.3</td>
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<tr>
<td>Si</td>
<td>NS</td>
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<td>NS 11.0</td>
<td>17.6</td>
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<td>–1.8 –1.5</td>
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<tr>
<td></td>
<td>Casp3 –2.0</td>
<td>–1.8 NS</td>
<td>NS NS</td>
<td></td>
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<td>Mucosal regeneration</td>
<td>Reg1 4.0</td>
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<td>NS NS</td>
<td></td>
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<tr>
<td></td>
<td>Pap1 2.0</td>
<td>1.6 NS NS</td>
<td>NS NS</td>
<td></td>
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<tr>
<td>Wnt/β-catenin pathway</td>
<td>Wnt5a NS</td>
<td>NS 2.2</td>
<td>3.7</td>
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<td></td>
<td>Wnt5b NS</td>
<td>NS 2.9</td>
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<td>Wnt7b NS</td>
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<td></td>
<td>Ctnnb NS</td>
<td>NS NS NS</td>
<td>NS NS</td>
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<td>Cdx1 –2.0</td>
<td>–1.5 –2.9</td>
<td>–2.3</td>
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<td>Cdx2 NS</td>
<td>NS –1.8</td>
<td>–1.9</td>
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<td></td>
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<td>C-myc NS</td>
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<td>Hedgehog pathway</td>
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<tr>
<td></td>
<td>Rrxα NS</td>
<td>NS –2.2</td>
<td>–2.1</td>
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<td>NS NS NS</td>
<td>NS NS</td>
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<td>–3.0</td>
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<td>Ppara NS</td>
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<td>ItgB3 –2.9</td>
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<td>Hgcl2 –2.6</td>
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Values represent the relative increase or decrease (negative) in expression for the indicated comparisons. RA, retinoic acid; Veh, vehicle; SHR, sham resected; RE, resected. Only significant changes (P < 0.05) are shown; NS indicates no significant difference.

changes in morphological parameters of adaptation. For example, proliferation increased ~1.20-fold in the duodenal-jejunal and ileal segments postresection. The addition of RA tended to further increase crypt cellular proliferation 1.14-fold in the duodenal-jejunal and ileum after resection (P < 0.01 and P = 0.05, respectively, by Tukey’s post hoc test) and 1.11-fold in the duodenal-jejunal postresection and 1.22-fold in the ileum (P < 0.05) following transection and reanastomosis.

**Cellular apoptosis.** Retinoic acid significantly inhibited apoptosis in the crypts of transected and resected rats (P < 0.05 for all segments; Fig. 5). The magnitude of the decrease in apoptotic index was ~52% in the duodenal-jejunal segment and 61% in the ileal segment of transected rats and 47% in both segments of resected rats. RA-induced inhibition of apoptosis was associated with significantly reduced ileal expression of Bax and caspase-3 mRNA levels (1.8-fold lower; Table 2). The reduction in Bax expression is consistent with data indicating that Bax is required for the resection-mediated enhanced apoptosis that is observed in some resection models (26, 47, 52, 53) The effects of surgery and RA treatment on apoptosis were not interactive.

**Enterocyte migration and components of the extracellular matrix.** Increased enterocyte migration occurs in the adapting remnant intestine postresection (50, 52, 53). RA treatment further increased migration by 40% in the remnant ileum following partial resection (Fig. 6). In a previous study, we showed that collagen IV expression was increased postresection, and this change as well as increases in enterocyte migration were inhibited in vitamin A-deficient rats (50). Thus the hypothesis that changes in components of the extracellular
matrix (ECM) may contribute to the increase in enterocyte migration and other proadaptive effects of RA administration was assessed using semiquantitative immunohistochemistry. Pericryptal collagen IV expression was independently enhanced by RA treatment and by surgical resection (Fig. 7). Compared with placebo, RA treatment increased collagen IV staining around crypts 1.64-fold in resected rats and 1.46-fold in sham-resected rats \( (P < 0.09 \text{ and } P < 0.05, \text{ respectively, by Tukey’s post hoc test}) \). Compared with sham-resected groups, resection increased pericryptal collagen IV staining 2.66-fold in vehicle-treated rats and 1.45-fold in RA-treated rats \( (P < 0.05 \text{ for both, by Tukey’s post hoc test}) \). In the basement membrane underlying villus enterocytes, there was no change in collagen IV staining attributable to resection and just a trend toward increased collagen IV staining in RA-treated rats \( (i.e., 1.51- \text{ to } 1.87\text{-fold increase, but } P \text{ for main effect of RA was NS, } P = 0.12) \).

Additional components of the ECM were also examined in the ileum of resected and transected rats. As discussed below, E-cadherin mRNA levels were significantly reduced by resection and by RA. mRNA levels for integrin receptor subunits, which bind to elements of the ECM \( (e.g., \text{collagen IV, laminin, and/or fibronectin}) \), were also determined. Both resection and RA significantly decreased integrin \( \beta_1 \) mRNA levels, and RA alone decreased integrin \( \alpha_5 \) levels, whereas the expression of integrins \( \alpha_2, \beta_1, \) and \( \beta_2 \) were not changed by either resection or RA. There were also no apparent changes attributable to resection or RA in immunostaining for laminin or fibronectin (data not shown).

Expression of genes differentially expressed in the adapting gut. To identify additional mechanisms contributing to the proadaptive effects of RA, we analyzed the expression of a cohort of genes (Table 2) shown by differential cloning and/or microarrays to be regulated in the remnant ileum postresection \( (9, 59) \). Genes reflecting enterocyte function including Na+/glucose cotransporter 1 \( (\text{Slc5a1 or SGLT1}) \), cellular retinol binding protein 2 \( (\text{Rbp2}) \), sucrase-isomaltase \( (\text{S}i) \), and the solute carrier family 2 facilitated glucose transporter \( (\text{Glut2, Slc2a2}) \) were significantly increased by resection but not by RA. Although resection also significantly induced expression of apolipoprotein A-IV \( (\text{ApoA-IV}) \), RA had the opposite effect.

On the basis of differential cloning, we also showed that several members of the Reg gene family were specifically induced in the adapting remnant ileum postresection at 48 h postoperation \( (9) \). In the current study, the expression of two of these, pancreatitis-associated protein \( (\text{Pap1, rat homolog of human Reg3}) \) and pancreatic stone protein \( (\text{RegI, rat homolog}) \)
of human RegIα) were studied at 2 wk postoperation, and both were significantly induced by RA treatment in transected and resected rats (PapI, 1.6- to 2.0-fold, P < 0.05; RegI, 2.9- to 4.0-fold, P < 0.05).

Expression of genes in the Wnt/β-catenin and Hedgehog pathways. Cross talk between RA and Sonic Hedgehog (Shh) and Wnt signaling pathways contributes to the regulation of gene expression mediated via homeobox (Hox), caudal-type homeobox (Cdx), and β-catenin-LEF-related transcription factors (e.g., see Refs. 11, 19, 32, 33, 40, 45, 51, 56). Thus the expression of several components of these pathways was examined. As shown in Table 2, β-catenin mRNA levels were not significantly changed by resection or RA, whereas E-cadherin and Cdx1 were significantly decreased by both. Wnt5a, Wnt5b, Wnt7b, Cdx2, Tcf4, Apc, and C-myc were all significantly decreased by resection but not by RA. There was no significant interaction between surgery and RA for any of these genes.

Sonic, Desert and Indian Hedgehog (Shh, Dhh, and Ihh) were all detected in the ileal mucosa of pre- and postoperative rats. Ihh, which was the most abundant, and Shh were both significantly decreased by resection but not by RA, whereas Dhh was unchanged by resection or RA. Both resection and RA significantly decreased the expression of the Hh receptors Ptc1 and Ptc2. Gli1 and Gli2, two downstream targets of Hh signaling, were expressed in the ileal mucosa. The expression of Gli1, which was >300 times more abundant than Gli2, was dramatically decreased by both resection and RA, whereas Gli2 mRNA levels were unchanged. Bone morphogenetic protein 4 (Bmp4) was also significantly decreased by resection (∼80% reduction at 2 w postoperation, P = 0.004 for surgical effect). There was a trend for RA to reduce Bmp4 expression, although the effect was not significant (P = 0.066).

DISCUSSION

Optimally, treatment of patients with short bowel syndrome will include strategies to increase the surface area and functional capacity of the residual small intestine. With the use of rodent small intestinal resection models, a variety of factors including glucagon-like peptide 2 (17), epidermal growth factor (12), insulin-like growth factor 1 (25), keratinocyte growth factor (20), and interleukin-11 (27) have been identified as putative augmenters of the adaptive response. Previous studies by our group demonstrating that small intestinal adaptation is inhibited in vitamin A-deficient rats (49, 50) suggested that endogenous vitamin A may be an important mediator of adaptation. Our work has also shown that a single dose of RA administered at the time of resection induced a rapid increase in crypt cell proliferation, but this effect was not sustained (58).

Thus the current study was undertaken to determine the therapeutic potential of chronically administered exogenous RA to augment the adaptive response and to address the mechanisms of any such effect. These studies provide convincing evidence that chronically administered RA can significantly enhance adaptation in the rat resection model and provide new data supporting its therapeutic potential in short bowel syndrome.

Analysis of serum and tissue retinoid levels confirmed that implantation of RA pellets 48 h preoperatively had the intended effect on RA levels. Serum RA levels were increased by 20% at the time of surgery. Furthermore, plasma and intestinal RA levels were sustained at increased levels throughout the 14-day postoperative period. Most pertinently, both all-trans RA and 9-cis RA were readily detectable in the intestine of RA- but not vehicle-treated rats. Thus, with this delivery method, the principal ligands for RA and rexinoid receptors (RA and 9-cis RA, respectively) were present at increased levels in the postoperative small intestine.

Exogenous RA stimulated the adaptive response by 2 wk post-70% small bowel resection as manifested by a significant increase in crypt depth, villus height, and intestinal surface area. The enlarged crypts and villi were due to adaptive hyperplasia and not to cellular hypertrophy. RA was also trophic in the intestine of control rats that were only subjected to transection and reanastomosis. In extraintestinal tissues, RA can influence cell proliferation, differentiation, migration, and fate by regulating RA-responsive genes specifying growth factors, growth factor receptors, and cell adhesion molecules (e.g., see Refs. 14, 57). Thus the contributions of these mechanisms to the prodaptive effects of RA were further explored.

Cyst and villus cellularity are determined by a complex balance among rates of crypt cell proliferation and apoptosis, epithelial cell migration, and anoikis (i.e., cell death initiated after loss of contact with the ECM or changes in interactions with the ECM) (15). Similarly, the morphological changes characteristic of the adaptive response after small bowel resec-
tion result from relative changes in these processes producing a new set point (for examples, see Refs. 16, 30, 43, 50, 52). Consistent with enhanced morphological adaptation, apoptosis was inhibited and crypt cell proliferation was stimulated by RA administration in both sham-resected and resected rats. In the resected rats, the effects of RA and resection on apoptosis and proliferation were additive. These results indicate that augmentation of the hyperplastic response by stimulation of crypt cell proliferation and inhibition of apoptosis is an important mechanism contributing to the proadaptive effects of RA.

The stimulation of crypt cell proliferation in the postresection remnant small intestine by RA is contrary to the inhibitory effects on intestinal, especially colonic, cell proliferation that have been attributed to RA in several in vitro and in vivo neoplasia models. The data are consistent, however, with our previous observation that acute administration of RA also stimulated the G1-to-S transition by 6 h following partial small bowel resection (58). The rapid onset of the RA effect suggests that the increased proliferation postresection is not solely linked to enhanced survival of proliferating crypt stem or...
transit cells. This interpretation is supported by the observation that adaptation-induced crypt cell apoptosis is inhibited in Bac null mice without stimulating crypt cell proliferation (47, 52).

Our data suggest that several pathways implicated in the regulation of intestinal crypt cell proliferation are modified by RA administration. For example, Hedgehog (Hh) signaling pathways play an important role in gut crypt and villus epithelial-mesenchymal interactions (41). Hh proteins (i.e., Shh, Ihh, and Dhh) when secreted from epithelial cells can bind to receptors (Ptc and Ptc2) in the underlying mesenchyme and thereby modulate Gli, Wnt, and Bmp transcription (5). We have shown that the adaptive response is associated with downregulation of components of the Hh signaling pathway (e.g., Hh, Ptc, Gli, and most Bmp genes) (53). The observation that RA treatment further suppressed expression of Ptc, Ptc2, and Gli2 and tended to reduce Bmp4 expression suggest that inhibition of Hh signaling is an additional mechanism contributing to the proadaptive effects of RA. The observed reduction in the expression of many components of the Wnt-\(\beta\)-catenin signaling pathway implies that the proadaptive response to reducing Bmp4 and Hh signaling is not mediated by stimulation of Wnt-\(\beta\)-catenin signaling.

In mice the increase in crypt cell proliferation that occurs following adaptation is accompanied by an increase in crypt cell apoptosis (16, 49, 50, 52) that is partially mediated via the extrinsic pathway linked via Bax to the mitochondrial pathway. In rats studied at 1 wk or later postresection, adaptation is associated with decreased or unchanged rates of apoptosis (6, 7, 55). The reduction in Bax expression observed in this study could be one of the mechanisms accounting for the lack of an increase in apoptosis in the adapting rat intestine. Studies in Bac\(^{-/-}\) mice suggest that modulation of apoptosis may be useful for enhancing adaptation (52). Conversely, increased apoptosis occurred concurrently with an attenuated adaptive response in vitamin A-deficient rats and in mice with impaired epidermal growth factor receptor signaling (18, 49, 50). Thus it is likely that modulation of apoptotic pathways could be a mechanism for enhancing adaptation in the rat as well.

In the current study, the proadaptive effects of RA were associated with a dramatic decrease in crypt cell apoptosis, and this occurred in the setting of reduced expression of Bax and caspase-3. Inhibited apoptosis was somewhat surprising, because induction of apoptosis and terminal differentiation more often result from administration of RA and/or synthetic analogs (for examples, see Refs. 13, 32, 42, 48). One of several exceptions to the paradigm of retinoid induction of apoptosis is the observation that apoptosis resulting from oxidant stress can be inhibited by retinoids. For example, oxidant (H\(_2\)O\(_2\))-induced apoptosis in renal mesangial cells is inhibited by RA via both nuclear receptor and indirect mechanisms (24, 63). In vitro and in vivo data suggest that oxidation of cellular redox pools in the intestine can inhibit cell growth and promote apoptosis (1, 21, 22, 34, 35). Quantification of lipid peroxidation and \(\alpha\)-tocopherol along the crypt-villus axis are consistent with increased oxidative stress in crypt stem cells and villus tip cells (29). Furthermore, following partial small bowel resection, the glutathione redox potential is shifted to a more oxidized state in the ileal remnant (62). Thus the observed prosurvival effects of RA postresection may be mediated by reducing oxidant stress in actively proliferating crypt cells. In addition, the prosurvival effects of RA postresection may be mediated by AKT-catalyzed phosphorylative inactivation of proapoptotic proteins (e.g., Bad). RA modulation of the Akt pathway has been demonstrated to affect both cell survival and cell death in many models. Akt signaling has also been shown to account for many of the prosurvival effects of PPAR\(\beta\)/\(\delta\) (e.g., see Ref. 8). The prosurvival effects of RA could also occur by this mechanism, since RA has been identified as a potent ligand for PPAR\(\beta\)/\(\delta\) (46). This hypothesis that the prosurvival effects of RA may be mediated via PPAR\(\beta\)/\(\delta\) and AKT is being investigated in our laboratory.

A variety of different mechanisms may mediate the stimulatory effects of RA postresection on epithelial cell migration, including mediation of epithelial-mesenchymal interactions. RA stimulated epithelial cell polarization and differentiation, including the expression of laminin and collagen IV, when added to cocultures of intestinal mesenchyme-derived cell lines and 14-day intestinal endoderm (58). RA treatment also accelerated villus outgrowth and epithelial differentiation in 14-day fetal rat intestine in organ culture or when implanted subcutaneously in nude mice (38). RA also stimulated epithelial cell polarization and differentiation, including the expression of laminin and collagen IV, when added to cocultures of intestinal mesenchyme-derived cell lines and 14-day intestinal endoderm (38). Thus retinoid modulation of ECM and mesenchymal epithelial interactions may contribute to intestinal adaptation. Direct evidence includes our observation that vitamin A deficiency blocked intestinal adaptation and markedly reduced laminin and collagen type IV expression after partial resection of the rat small intestine (49, 50). In the current studies, RA administration stimulated collagen IV deposition in the ECM underlying intestinal crypts. This effect may be mediated by suppressing collagenase expression (37). RA also inhibited expression of integrin receptor subunits \(\beta\) and \(\alpha\). These integrins have been implicated as modulators of enterocyte migration (39, 61). For example, overexpression has been associated with inhibition of enterocyte migration in an experimental rat model of necrotizing enterocolitis (39). Thus retinoid modulation of the expression of integrins and other components of the ECM may contribute to the increased enterocyte migration rates postresection and post-RA treatment.

The pleiotropic effects of RA also result from its ability to regulate the expression, secretion, and/or function of multiple growth factor genes and to differentially regulate transcriptional programs activated by RARE, AP-1, and \(\beta\)-catenin-LEF/ TCF (for example, see Ref. 11). Thus we examined the expression of several genes previously identified to be differentially expressed in the adapting intestine (see Table 2 and Ref. 9). The expression of several RAR, RXR, and PPAR subtypes was reduced in the adapting intestine, thus suggesting that relative changes in the ratio of receptor subtypes can contribute to the adaptive response. However, RA did not independently affect the expression of RARs or RXRs, thus suggesting that the proadaptive effects of RA are not dependent on autoregulatory changes in these receptors. RA significantly enhanced expression of both Pap1 and Reg1 in the adapting gut. Pap1 and Reg1 are representative members of the Reg gene family of C-type lectin secretory proteins. We had previously cloned several Reg-related proteins on the basis of preferential induction in the remnant gut postresection (9). Although the role of these proteins in the small intestine has not been systematically...
studied, members of the Reg family are thought to promote pancreatic and hepatic regeneration after injury. There is also evidence that PAP, which is abundantly expressed in the small intestine, stimulates proliferation of intestinal epithelial cells (31). Reg1, which is thought to be an important mediator of pancreatic islet cell regeneration, is also expressed in small intestinal crypt cells. Crypt cell proliferation and enterocyte migration were markedly reduced in Reg1 knockout mice, suggesting that Reg1 is required for generation and maintenance of the small intestinal epithelium (36). These data and observations suggest that the significant induction of Pap1 and Reg1 by RA in the remnant intestine may contribute to the proadapative effects of RA on proliferation, apoptosis, and enterocyte migration.

In summary, these studies are the first to establish that administration of RA can significantly augment the small intestinal adaptive response after resection. The proadapative phenotype can be attributed to the discovery that RA can stimulate small intestinal crypt cell proliferation while inhibiting apoptosis and inducing changes in the extracellular matrix that enhanced enterocyte migration. These studies suggest that vitamin A administration to short bowel syndrome patients with low or normal vitamin A stores may enhance the intestinal absorptive surface area. Thus natural dietary or synthetic retinoids may provide a relatively inexpensive, well-tolerated adjunct to the treatment of patients with short bowel syndrome.

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