Increased apoptosis and accelerated epithelial migration following inhibition of hedgehog signaling in adaptive small bowel postresection

Yuzhu Tang, Elzbieta A. Swietlicki, ShuJun Jiang, Kim K. Buhman, Nicholas O. Davidson, Linda C. Burkly, Marc S. Levin, and Deborah C. Rubin. Increased apoptosis and accelerated epithelial migration following inhibition of hedgehog signaling in adaptive small bowel postresection. Am J Physiol Gastrointest Liver Physiol 290: G1280–G1288, 2006. First published January 26, 2006; doi:10.1152/ajpgi.00426.2005.—The intestinal epithelium undergoes a marked adaptive response following loss of functional small bowel surface area characterized by increased crypt cell proliferation and increased enterocyte migration from crypt to villus tip, resulting in villus hyperplasia and enhanced nutrient absorption. Hedgehog (Hh) signaling plays a critical role in regulating epithelial-mesenchymal interactions during morphogenesis of the embryonic intestine. Our previous studies showed that blocking Hh signaling in neonatal mice resulted in increased small intestinal epithelial crypt cell proliferation and altered enterocyte fat absorption and morphology. Hh family members are also expressed in the adult intestine, but their role in the mature small bowel is unclear. With the use of a model of intestinal adaptation following partial small bowel resection, the role of Hh signaling in the adult gut was examined by determining the effects of blocking Hh signaling on the regenerative response following loss of functional surface area. Hh-inactivating monoclonal antibodies or control antibodies were administered to mice that sustained a 50% intestinal resection. mRNA analyses of the preoperative ileum by quantitative real-time PCR revealed that Indian hedgehog was the most abundant Hh family member. The Hh receptor Patched was more abundant than Patched 2. Analyses of downstream targets of Hh signaling demonstrated that Gli3 was twofold more abundant than Gli1 and Gli2 and that bone morphogenetic protein (BMP)2 was most highly expressed compared with BMP1, -4, and -7. Following intestinal resection, the expression of Hh, Patched, Gli, and most BMP genes was markedly downregulated in the remnant ileum, and, in anti-Hh antibody-treated mice, expression of Patched 2 and Gli 1 was further suppressed. In Hh antibody-treated mice following resection, the enterocyte migration rate from crypt to villus tip was increased, and by 2 wk postoperation, apoptosis was increased in the adaptive gut. However, crypt cell proliferation, villus height, and crypt depth were not augmented. These data indicate that Hh signaling plays a role in adult gut epithelial homeostasis by regulating epithelial cell migration from crypt to villus tip and by enhancing apoptosis.

intestinal adaptation; bone morphogenetic proteins; Patched

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The small intestine contains a rapidly proliferating epithelium that can mount a robust adaptive response following loss of functional small bowel surface area. This response is characterized by a marked increase in crypt cell proliferation resulting in villus hyperplasia, increased enterocyte migration from crypt to villus tip, and enhanced nutrient absorption (12, 20, 37). We and others have shown that enterocyte apoptosis is also modified during the adaptive response (13, 37). The molecular mechanisms that regulate this unique adaptive response are still being clarified.

The hedgehog (Hh) signaling pathway plays an important role in epithelial-mesenchymal interactions in gut morphogenesis and epithelial cell proliferation (11, 26, 27). The mammalian Hh family includes Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Hh proteins are produced in epithelial cells and interact with underlying mesenchymal/stromal cell receptors such as Patched (Ptc) and Ptc 2 (5, 7, 8). Interaction of Shh with Ptc relieves Ptc of its tonic inhibition on Smoothened (Smo), a second membrane receptor, which leads to activation of a complex signaling cascade that results in the translocation of Gli transcription factors to the nucleus (8, 22, 42). The importance of Hh signaling in gut morphogenesis and intestinal cellular proliferation has been demonstrated by studies of Shh−/− and Ihh−/− mice (17, 26). Shh−/− mice die either before or soon after birth with multiple anomalies including tracheoesophageal fusion, reduced gut smooth muscle, intestinal malrotation, annular pancreas, intestinal transformation of the stomach, and duodenal stenosis. Ihh−/− mice also die at or just after birth, and intestines show reduced epithelial stem cell proliferation and differentiation and exhibit some features of Hirschsprung disease (26).

Hh proteins are expressed not only during embryonic development but also in the neonatal and adult small bowel (23, 40). To determine the role of Hh in neonatal and adult mice, strategies have been developed to overcome the embryonic lethality of the null defect. We (4) and others (43, 44) have infused inactivating monoclonal antibodies or used cyclopamine (31, 39, 41) to block Hh signaling in neonatal (44) and adult (4, 31, 41) mice. Also, Hh signaling was inhibited by embryonic day 12 in transgenic mice in which the gut-specific villin promoter was utilized to overexpress Hh-inhibitory protein in the intestine (18). These studies showed that blocking Hh signaling in the fetus or neonate leads to increased crypt cell proliferation, crypt-villus axis structural anomalies, and alterations in enterocyte morphology. In addition, reduced Hh signaling resulted in mislocalization of intestinal subepithelial myofibroblasts along the crypt-villus axis (18), suggesting that Hh signaling pathways regulate anchoring and migration of...
these cells in the epithelium. In contrast, others have shown that activating Hh signaling increases growth of pancreatic, biliary tract, and other upper digestive cancers (3, 38). Thus the role of Hh signaling in regulating proliferation in regenerating epithelium in the mature small intestine remains unclear.

To determine the role of Hh signaling in adult small intestinal epithelial cell proliferation and the regenerative, adaptive response, we studied the effects of blocking Hh signaling in the remnant adaptive ileum of mice that were subjected to partial small intestinal resection. Our results suggest that Hh signaling pathways regulate enterocyte migration and apoptosis in the adaptive response following resection but do not enhance the adaptive increase in crypt cell proliferation.

MATERIALS AND METHODS

Mice and experimental design. All animal experimentation was conducted in conformity with the American Physiology Society’s “Guiding Principles in the Care and Use of Animals,” approved by the Washington University Animal Studies Committee, and performed under Institutional Animal Care and Use Committee-approved protocols. Male BALB/cJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were housed for several weeks prior to surgery in the Washington University School of Medicine barrier facility in a 12:12-h light-dark cycle. Mice received a regular, low-fat, and nonpurified diet containing protein, carbohydrates, and 4.5, 20.0, or 36.8 g/100 g from fat, respectively (PicoLab rodent diet 20, Ralston Purina). At 12 wk of age, mice were prepared for intestinal resection as previously described (37). Mice were introduced to the AIN93G liquid diet containing 4.0, 14.1, and 70.7 g/100 g from fat, protein, and carbohydrate, respectively (Dyets) for 5 days prior to surgery, and the regular diet was removed 1 day prior to surgery. Mice were subjected to 50% small intestinal resection (from 2–3 cm distal to the ligament of Treitz to 8 cm proximal to the cecum), followed by end-to-end anastomosis of the remaining jejunum with the distal ileum. The resected intestine was saved for histological and gene expression analysis. Gentamicin (0.2 mg in 0.5 ml saline) was administered intraperitoneally and postoperative analgesia was obtained by using buprenorphine (0.3 mg/kg) subcutaneously. Mice were paired by weight into four groups (n = 8–9 mice group) and received either anti-Hh monoclonal antibody (MAb) SE1 or isotype-matched control MAb 1E6 (6 mg/kg body wt administered intraperitoneally every other day) for 1 or 2 wk. The anti-Hh MAb SE1 has been shown to block Hh signaling by binding to all Hh proteins (4, 43). The SE1 antibody, when administered at the dose chosen for the present study (6 mg/kg), has been shown to block Hh activity in vitro and in vivo (42), inhibiting both Shh-induced differentiation of C3H10T1/2 cells and hair formation in adult mice. Mice were killed, and the remnant jejunum and ileum were removed and either

Epithelial cell migration was measured using a double-labeling technique with 5-BrDU, as previously described (37). Mice were doubly injected with 5-BrDU (4.8 mg/kg) at 49.5 h and with 5-BrDU (120 mg/kg) at 1.5 h prior to death. Using these dosages and time intervals, two zones of BrDU-labeled cells were detected, representing newly labeled cells (from the 90-min injection) and migrating cells (representing the cells labeled 48 h earlier). There was no significant overlap of labeled cells, and labeled cells were not present at the villus tips, thus eliminating the possibility of falsely low rates of migration. Sections were stained for BrDU, and each cell position located from the base of the crypt to the villus tip was scored for 5-BrDU labeling and plotted against cell position. Migration rates were calculated as the differences between the leading-edge, median-peak labeling positions on the crypt and villus distribution curves (36).

Quantification of apoptosis. Identification and quantification of apoptotic bodies were performed using two techniques including staining for activated caspase 3 (Cell Signaling Technology, Beverly, MA) and routine morphological assessment following hematoxylin and eosin staining of sections. Morphological changes included the presence of nuclear condensation, perinuclear clearing, and cell shrinkage (19, 37). The apoptotic index was calculated as the numbers of apoptotic bodies per 100 crypt cells. Only full-length crypts were used for analysis.

Immunohistochemical analyses. Immunohistochemical staining was performed for E-cadherin and collagen type IV on the formalin-fixed, paraffin-embedded proximal ileum from anti-Hh MAb- and control MAb-treated mice. Antibodies used were rabbit anti-human E-cadherin (1:10; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-human and bovine placental collagen type IV (1:100; Chemicon International, Temecula, CA) at 4°C overnight. Secondary antibodies were anti-rabbit or anti-goat Rhodamine red (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were deparaffinized and treated with Nuclear Decloaker at 18 psi for 3 min (Biocare Medical, Walnut Creek, CA), and then primary antibodies were applied. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Staining intensity was quantified by ScionImage (36).

Quantitative real-time RT-PCR for gene expression. Primers for each gene were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA). Genes and primers are summarized in Table 1.

Total RNA was extracted from the full-thickness intestine or stomach by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and then cleaned by DNA-free (Ambion, Austin, TX) to remove unwanted DNA. cDNA was synthesized by reverse transcription of 2 μg total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen Life Technology). Real-time PCRs were performed on an ABI Prism 7000 sequence detection system with default settings, using 2× SYBR green master mix (Applied Biosystems), per the manufacturer’s directions. Relative gene expression was determined using the comparative cycle threshold method as per Applied Biosystems user bulletin no. 2.

Immunoblot analysis. Immunoblot analysis was performed to quantify the expression of bone morphogenic protein 2 (BMP2) and integrins-β1 and -α3. Immunoblots were performed as previously described (37, 45). Briefly, intestinal extracts were prepared, and proteins were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred onto PVDF-PLUS membranes. Immunoblots were incubated with mouse MAb to integrin-β1, integrin-α3 (1:2,500; BD Biosciences), integrin-α1 (1:250, BD Biosciences), or goat polyclonal anti-human BMP2 antibody (1:100, Santa Cruz Biotechnology), which cross-reacts with the mouse. Immunoblots were then incubated with horseradish peroxidase-conjugated anti-IgG antibodies (1:10,000; Amersham Biosciences, Piscataway, NJ) and developed with chemiluminescent peroxidase substrate (ECL Western blotting kit, Amersham Biosciences).
Data analysis. Changes in villus height, crypt depth, proliferation, apoptosis, migration, and gene-expression levels were assessed by two-way ANOVA and the Tukey post-hoc test (Sigma Stat 2.03, Jandel Scientific Software, Chicago, IL) with main factors of post-resection time and antibody treatment. The morphometric adaptive response was also evaluated by paired t-test, comparing the postoperative jejunum and ileum with their preoperative fragments. Differences were considered significant at a P value ≤0.05.

RESULTS

Expression of Hh and downstream transcriptional target genes in ileum and stomach. To determine expression patterns of Hh family members and the transcriptional targets of Hh signaling, quantitative real-time RT-PCR was performed on the proximal ileum and stomach to detect steady-state Hh, Ptc1, Gli1, and BMP family mRNA levels. The proximal ileum was chosen because this is the region of the gut that shows the most robust adaptive response following resection (12, 29). Relative expression levels of Shh, Ihh, Dhh, Ptc1, Ptc2, Gli1, -2, -3 and BMP1, -2, -4, and -7 mRNAs were first determined in the normal, unoperated proximal ileum, and in the stomach as a control (Fig. 1), because expression of these genes in the stomach is well characterized (3, 40, 41). In the proximal ileum, in contrast to the stomach, Shh mRNA could not be detected. However, Ihh mRNA was readily detected and was ~12.9-fold more abundant than Dhh mRNA levels. Ptc1 was more abundant than Ptc2 in both tissues. Gli3 was the most abundantly expressed of the Gli transcription factors in the stomach and ileum, and BMP2 was most abundant compared with BMP1, -4, and -7 in both tissues.

Suppression of Hh signaling pathways following small bowel resection. To determine the role of Hh signaling in the intestinal adaptive response following loss of functional surface area, 50% small intestinal resections were performed on adult mice. Beginning on the day of surgery and every other day thereafter, mice were treated either with the monoclonal inactivating antibody 5E1 or isotype-matched control antibody 1E6 for 1 or 2 wk.

Our initial survey, using mice receiving only the control antibody, revealed inhibition of Hh signaling in the postoperative “remnant” ileum (Fig. 2). By 1 wk after resection, expression of Ihh and Dhh mRNA was decreased to only 33% and 13% of preoperative control ileal mRNA levels, respectively. Ptc1 and Ptc2 mRNA levels were suppressed to 23% and 17% of preoperative ileal levels, respectively. Gli1, -2, -3 mRNA levels in the adaptive ileum were all decreased, to 15%, 13%, and 19% of preoperative mRNA levels, respectively. BMP4 was suppressed to 16% of preoperative ileal levels, and BMP1, -2, and -7 mRNA levels were 37%, 26%, and 60% of normal levels, respectively.

At 2 wk following resection, Hh mRNA levels remained suppressed. Ptc1 and BMP4 mRNA levels increased in the adaptive 2-wk ileum compared with 1 wk after resection but were still suppressed compared with the control, preoperative ileum. Ptc2, Gli1, -2, -3, and BMP1 and -2 mRNAs remained suppressed. Thus intestinal resection results in decreased Hh signaling in the remnant gut.

Treatment with anti-Hh MAb 5E1 further suppresses Hh signaling in the adaptive intestine. Previous studies have shown that infusion of MAb SE1 blocks Hh signaling in the intestine and other organs (4, 44). To verify these effects in the mouse model of gut adaptation following intestine resection, the expression of Hh genes and their transcriptional targets were quantified by quantitative RT-PCR (Fig. 3A) in the adapting ileum after 1 or 2 wk of treatment with anti-Hh MAb.

Following infusion of monoclonal inactivating anti-Hh antibodies for 1 wk after resection, Ptc2 and Gli1 mRNA levels were reduced compared with control antibody–treated resected mice (2.3- and 4.5-fold, respectively). However, BMP2 and -7 mRNA levels increased (2- and 1.6-fold, respectively). Yet, by 2 wk after resection, continued infusion of anti-Hh antibodies suppressed ileal Ptc2, Gli1, BMP1, and BMP2 mRNA expression. BMP4 expression, although increased at 2 wk compared with 1 wk after resection, remained suppressed compared with the preoperative ileum. To correlate mRNA levels with protein expression, immunoblot analysis was performed to determine BMP2 levels in resected control and resected anti-Hh MAb–treated intestines. The changes in BMP2 protein expression

Table 1. Summary of genes and primers

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<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Forward Primers (5’-3’)</th>
<th>Reverse Primers (5’-3’)</th>
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<tr>
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in the anti-Hh-treated adaptive proximal ileum compared with the control resected intestine; also, apoptosis was increased in anti-Hh-treated intestine at 2 wk compared with 1 wk after resection (Fig. 4). No change in the number of villus-associated apoptotic cells was seen. Despite the increase in crypt apoptosis, there was no apparent change in crypt cell numbers. In concert, an increase in enterocyte migration was noted in the anti-Hh-treated mice compared with control antibody-treated mice at 1 and 2 wk after resection (Fig. 5). In addition, the rate of epithelial cell migration from crypt to villus tip was increased at 2 wk after resection compared with the rate at 1 wk postresection. Epithelial cell migration was most rapid in the Hh antibody-treated mice at 2 wk following resection. No change in crypt cell proliferation was noted in the MAb-treated adaptive ileum compared with the control adaptive ileum at either 1 or 2 wk following resection (Fig. 6A). Villus height and crypt depth were not further increased by blocking Hh signaling (Fig. 6B).

To determine whether the expression of basement membrane proteins thought to be involved in regulating epithelial cell migration was altered in Hh-treated mice, real-time PCR, immunoblot, and immunohistochemical analyses were performed to evaluate expression of collagen type IV and E-cadherin. Decreased expression of the collagen IV α5-subunit was found by real-time PCR, following 2 wk of antibody treatment after resection compared with control antibody infusion (Fig. 7A) but otherwise, no quantitative change in collagen type IV or E-cadherin protein levels and no alteration in immunostaining intensity or distribution were noted in the small bowel of Hh antibody-treated mice (Fig. 7, B–I).

Blocking Hh signaling increases both apoptosis and enterocyte migration during gut adaptation. To determine the blockage of Hh signaling on the intestinal adaptive response following loss of small bowel surface area, morphometric studies were performed including quantification of intestinal crypt cell proliferation, apoptotic rate, enterocyte migration rate, and measurement of villus heights and crypt depths. Apoptosis in the crypts was increased by 2 wk after resection (Supplemental Fig. S1) mirrored the changes in mRNA expression noted in Fig. 3. As a control, expression of Hh-signaling pathway genes were examined in the stomach following antibody infusion for 1 and 2 wk (Fig. 3B). Expression of the Ihh, Dhh, and BMP genes did not change. Similar to the intestine, Ptc2 and Gli1 mRNA levels were decreased. After 2 wk of antibody infusion, Shh and Ptc expression were also decreased.

**Fig. 1.** Relative Hh, Ptc, Gli, and BMP mRNA expression in normal, preoperative ileum and stomach. Quantitative real-time RT-PCR was performed as per MATERIALS AND METHODS. A: relative Hh and Ptc mRNA expression levels in ileum and stomach. B: relative Gli and BMP mRNA expression levels in intestine and stomach (n = 6 mice for ileum and 4 for stomach). Shh, Sonic hedgehog (Hh); Ihh, Indian Hh; Dhh, Desert Hh; Ptc, Patched; BMP, bone morphogenetic protein.

**Fig. 2.** Hh, Ptc, Gli, Smo, and BMP mRNA expression is suppressed in postoperative compared with preoperative ileum at 1 or 2 wk (W) following resection (RE). Quantitative real-time RT-PCR was performed as per MATERIALS AND METHODS. Postoperative mRNA levels for all genes are expressed as the percentage of preoperative expression for each group (n = 7–8 mice/group). *P < 0.05, preoperative vs. postoperative. #P < 0.05, 2 wk vs. 1 wk following resection. Smo, smootherned.

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1 The Supplemental Material for this article (Supplemental Fig. S1) is available online at http://ajpgi.physiology.org/cgi/content/full/00426.2005/DC1.
Integrins-α3 and -β1 have been shown to be involved in enterocyte migration in necrotizing enterocolitis (25). We therefore examined the ileal expression of these integrins by immunoblot and found that integrin-β1 was increased following resection and administration of the anti-Hh MAb for 2 wk compared with control antibody administered for 2 wk after resection (Fig. 8). Integrin-α3 expression levels were unchanged at both time points (data not shown).

DISCUSSION

Hh signaling pathways play a critical role in patterning and morphogenesis of the gut during fetal and neonatal life (9, 17, 21, 26, 35, 44). Hh proteins are also expressed in the mature gastrointestinal tract, but their function in the adult small bowel remains unclear. The present studies indicate that 1) monoclonal inactivating antibodies are effective in blocking Hh signaling in the adult mouse intestine; 2) Hh signaling is repressed in the adaptive intestine postresection; and 3) further repression of Hh signaling by MAb treatment following intestinal resection results in enhanced epithelial cell migration and apoptosis but does not increase the gut’s proliferative adaptive response to loss of functional small bowel surface area.

Following intestinal resection, we found that Hh signaling pathways are inhibited in the adapting remnant ileum. Following resection, a well-defined series of morphometric changes occur, including increases in crypt cell proliferation, epithelial cell migration, and crypt cell apoptosis, resulting in villus and crypt cell hyperplasia and lengthening of the crypt-villus axis. The inhibition in Hh signaling may be required for initiation of one or more of these aspects of the adaptive response. Our data suggest that Hh signaling primarily affects epithelial cell migration and apoptosis because both epithelial cell migration and apoptotic rates were further increased in antibody-treated mice following resection. Inhibiting Hh signaling thus may be required for the promigratory, proapoptotic pathways activated in the remnant gut following resection.

In a variety of cancer models including skin, colon, and prostate cancer, activation of Hh signaling leads to inhibition of apoptosis, and blockade with cyclopamine or other inhibitors of Hh signaling enhances apoptosis and decreases tumor size (16, 24, 28, 30, 33, 46). Our results showing increased crypt cell apoptosis in Hh antibody-treated adaptive intestine are consistent with these observations, i.e., that blocking hedgehog signaling leads to increased apoptotic cell death. Following small bowel resection, there is increased crypt cell apoptosis in the adapting mouse intestine (13, 37), presumably as a protective mechanism to eliminate damaged stem cells following injury. This increase was accompanied by increased expression of Fas/Fas ligand (37) and was blocked in Bax-null mice (34, 37), implicating the extrinsic death receptor and Bax/Bcl2 pathways in this response. Others have shown that Fas/Fas ligand apoptotic pathways are regulated by Hh, e.g., inhibition of Hh signaling in basal cell carcinoma cell
lines leads to increased apoptosis and increased Fas expression (1). This increase in apoptosis could be blocked by anti-Fas ligand antibodies.

In our studies, blockade of Hh signaling also further increased the rate of epithelial cell migration from crypt to villus tip following resection. The mechanisms underlying the observed increase in epithelial cell migration are unclear. Hh signaling controls morphogenetic cell movements during embryogenesis (6). Expression of several basement membrane proteins thought to be involved in regulating epithelial cell migration, including collagen type IV (2, 32, 36) and E-cadherin (10, 14, 15), were unchanged in Hh MAb-treated adaptive intestine, as measured by immunoblot and immunohistochemical analyses. mRNA levels encoding the collagen IV α3-subunit of collagen IV were modestly decreased in the anti-Hh MAb-treated, adaptive intestine following 2 wk of treatment, but it is unlikely that this change alone could account for the increase in enterocyte migration. Studies of neonatal intestine in which Hh signaling has been blocked by overexpression of hedgehog inhibitory protein driven by the villin promoter show that the distribution and migration of subepithelial myofibroblasts was altered, with mislocalization of these cells in the lamina propria of the villus tips (18).

Although the epithelial cell migration rate from crypt to villus tip increased in anti-Hh MAb-treated mouse gut, the organization of the crypt and villus-associated cells and lamina propria was unchanged. No alteration in the crypt-villus distribution of these cells in the lamina propria of the villus tips (18).

The expression of integrin-β1 was increased by administration of anti-Hh MAb for 2 wk after resection compared with administration of control MAb. Integrins-α3 and -β1 have been proposed to be involved in regulating enterocyte migration, as determined in an experimental model of necrotizing enterocolitis (25). In the necrotizing enterocolitis model, expression of both integrins increased, associated with a decrease in enterocyte migration. In our model, integrin-α3 expression did not change; thus it is possible that the ratio of integrin-α3 to -β1 may determine migration rate or other factors may interact with integrin-β1 to produce the increase in migration found in our model of adaptation following resection.

Hh signaling was inhibited in the adaptive intestine coincident with a marked increase in crypt cell proliferation. In the colon, Hh acts as an antagonist of Wnt signaling, an important pathway regulating gut epithelial proliferation (39). It is possible that downregulation of Hh signaling is required in the adaptive gut to permit an increase in Wnt signaling in the crypt, thereby resulting in increased crypt cell proliferation. Although further inhibition of Hh signaling by infusing blocking antibodies had no effect on proliferation in the adaptive adult gut, a critical level of inhibition may have already been achieved; thus an additional decrease in signaling had no effect. Hh signaling effects on gut epithelial proliferation and differentiation appear to be regulated in a com-

![Graph showing epithelial cell migration rate](image)

Fig. 5. Epithelial cell migration rate is increased in the adapting, anti-Hh MAb-treated mouse intestine (H) compared with control antibody-treated mice (C). Mice were injected with 5-bromodeoxyuridine (5-BrdU) at 49.5 h and 90 min prior to death, as described in MATERIALS AND METHODS. Migration distance of median cell position after 48 h was measured from crypt to villus as per MATERIALS AND METHODS. Data are means ± SE. Open bars are control mice; filled bars are anti-Hh-treated mice. Migration was significantly faster in anti-Hh MAb-treated mouse intestine compared with control MAb-treated mice and at 2 wk compared with 1 wk after resection. *P < 0.05, anti-HH vs. control. #P < 0.05, 2 wk vs. 1 wk. n = 5 mice group.

![Graph showing crypt cell proliferation rates](image)

Fig. 6. Crypt cell proliferation rates and morphometry of the adaptive crypt and villus following treatment with anti-Hh MAb or control MAb. Intestinal resections were performed, and mice were treated with anti-Hh MAb or control MAb for 1 or 2 wk after resection. A: mice were injected with 5-BrdU and killed 90 min later to measure crypt cell proliferation rates, per MATERIALS AND METHODS. B: morphological adaptation was quantified by comparing crypt depths and villus heights in adaptive jejunum (JEJ) and ileum (PI), as per MATERIALS AND METHODS. Crypt depth, villus height, and crypt cell proliferation rates increased following surgery alone, but anti-Hh MAb-treatment did not further enhance this response. Hatched bars, preoperative; open bars, resection plus control antibody; closed bars, resection plus anti-Hh MAb. Data are means ± SE. *P < 0.05, postoperative vs. preoperative. #P < 0.05, 2 wk vs. 1 wk. n = 8 mice group.
plex developmental-stage and region-specific manner. Shh−/− mice (which die at or shortly after birth) have increased proliferation in the stomach and duodenal epithelium during fetal life, yet Ihh−/− mice have decreased intervillus epithelial (stem) proliferation in fetal small bowel and exhibit small and large bowel dilation (17, 26). In contrast, in the fetal colon (embryonic day 16.5), Ihh−/− mice have increased cell proliferation compared with the wild type (39). Furthermore, gut-specific inhibition of Hh signaling in transgenic mice overexpressing Hh-inhibitory protein driven by the villin promoter resulted in increased gut epithelial cell proliferation, inhibition of differentiation, and aberrant crypt-villus axis formation in neonates (18). Also, colonic epithelial proliferation is increased in adult rats treated with cyclopamine, and, as mentioned above, in vitro data in colon cancer cell lines suggest that Hh signaling antagonizes the Wnt/β-catenin pathway (39). Finally, the complexity of Hh signaling in the gut is further demonstrated by data that indicate that activation of Hh signaling enhances the growth of upper digestive tract tumors (3, 38) including those of the pancreas, biliary tract, esophagus, and stomach. Thus the role of Hh signaling in the normal adult gut and in digestive cancers is complex and still needs to be clarified. Future studies in which mice are engineered to overexpress Hh in the gut following resection may help determine the importance of Hh in regulating small bowel proliferation in normal mucosa.

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Fig. 7. Collagen IV subunit and E-cadherin mRNA expression in adaptive ileum. Relative mRNA levels (expressed as fold change compared with preoperative ileum) encoding col4a1, col4a2, col4a5, and E-cadherin were quantified by real-time PCR and presented as fold change compared with preoperative ileum. Open bars, 1 wk postresection plus control antibody; shaded bars, 1 wk postresection plus anti-Hh MAb; hatched bars, 2 wk postresection plus control antibody; solid bars, 2 wk postresection plus anti-Hh MAb. There was no difference in col4a1, col4a2, and E-cadherin gene expression among all four groups, and col4a5 was decreased by 2 wk of treatment with anti-Hh MAb and there was an interactive effect of anti-Hh and time of resection. #P < 0.05, 2 wk vs. 1 wk. For col4a5 (box), P < 0.05 for interaction between antibody treatment and time of resection. n = 7 for 1 wk control, 5 for 1 wk anti-Hh, 8 for 2 wk control, and 8 for anti-Hh. B–F: immunohistochemical analysis of collagen IV collagen and E-cadherin expression in adaptive, anti-Hh- or control MAb-treated intestine. Mice underwent 50% small bowel resection as above. B–E: detection of collagen IV in 1 wk (B) or 2 wk (D) control-treated intestine or 1 wk (C) or 2 wk (E) anti-Hh MAb-treated intestine. Bright red fluorescence is noted in the lamina propria. F–I: detection of E-cadherin in 1 wk (F) or 2 wk (H) control-treated intestine or 1 wk (G) or 2 wk (I) anti-Hh MAb-treated intestine. Red fluorescence in enterocytes indicates the presence of E-cadherin. Administration of anti-Hh antibody did not change the pattern of expression of either protein.

Fig. 8. Increased integrin-β1 expression in adapting intestine following anti-Hh MAb administration. Intestinal resections were performed and mice were treated with anti-Hh MAb or control MAb for 1 or 2 wk after resection. Expression of integrin-β1 was quantified by immunoblot per MATERIALS AND METHODS. *P = 0.02, control vs. anti-Hh MAb at 2 wk after resection.
REFERENCES


