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Hedgehog is an anti-inflammatory epithelial signal for the intestinal lamina propria

William J. Zacharias¹, Xing Li², Blair B. Madison³, Katherine Kretovich¹, John Y. Kao⁴, Juanita L. Merchant^{4,5}, and Deborah L. Gumucio¹

¹Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, Michigan

²Bioinformatics Program, University of Michigan Medical School, Ann Arbor, Michigan

³Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

⁴Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

⁵Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan

Abstract

Background and Aims—Epithelial Hedgehog (Hh) ligands regulate several aspects fetal intestinal organogenesis and emerging data implicate the Hh pathway in inflammatory signaling in adult colon. Here, we investigated the effects of chronic Hh inhibition *in vivo* and profiled molecular pathways acutely modulated by Hh signaling in the intestinal mesenchyme.

Methods—The progression of inflammatory disease was characterized in a bi-transgenic mouse model of chronic Hh inhibition (VFHhip). In parallel, microarray and bioinformatic analyses (GO term overrepresentation analysis, hierarchical clustering, and MeSH term filtration) were performed on isolated cultured intestinal mesenchyme acutely exposed to Hh ligand.

Results—Six to ten month old VFHhip animals exhibited villus smooth muscle loss and subsequent villus atrophy. Areas of villus loss become complicated by spontaneous inflammation and VFHhip animals succumbed to wasting and death. Phenotypic similarities were noted between the VFHhip phenotype and human inflammatory disorders, especially human celiac disease. Microarray analysis revealed that inflammatory pathways were acutely activated in intestinal mesenchyme cultured in the absence of epithelium, and the addition of Hh ligand alone was sufficient to largely reverse this inflammatory response within 24 hours.

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Corresponding Author: Deborah L. Gumucio, Ph.D. University of Michigan Medical School Department of Cell and Developmental Biology 109 Zina Pitcher Place, 2045 BSRB Ann Arbor, MI 48109-2200 Phone: 734-647-0172 FAX: 734-647-9559 dgumucio@umich.edu.

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Conclusions—Hh ligand is a previously unrecognized anti-inflammatory epithelial modulator of the mesenchymal inflammatory milieu. Acute modulation of Hh signals results in changes in inflammatory pathways in intestinal mesenchyme, while chronic inhibition of Hh signaling in adult animals leads to spontaneous intestinal inflammation and death. Regulation of epithelial Hh signaling may be an important mechanism to modulate tolerogenic vs. pro-inflammatory signaling in the small intestine.

Keywords

Hedgehog signaling; Inflammatory bowel disease; celiac disease; myeloid cells; transgenic mouse model; epithelial/mesenchymal crosstalk

Introduction

Functional intestinal immunity requires a precise balance of pro-inflammatory and tolerogenic influences to both protect against infectious disease and prevent aberrant inflammation. When inappropriate pro-inflammatory signals dominate, as is the case in Inflammatory Bowel Disease (IBD) and celiac disease, significant tissue damage results, leading to significant patient morbidity. While IBD is thought to result from aberrant chronic activation of the immune system in response to luminal flora¹, celiac disease involves an immune response to dietary gluten. In both cases, the disease etiology involves a complex interplay between luminal agents, the intestinal epithelium and the immune elements within the underlying mesenchymal compartment. Indeed, through secretion of molecules such as thymic stromal lymphopoietin (TSLP), the epithelium is a critical modulator of the mesenchymal inflammatory response²⁻⁴. However, other epithelial modulators of inflammation must exist, since dendritic cells isolated from mice null for the TSLP receptor do not show defects in their ability to activate regulatory T cells⁵ and TSLP is not capable of completely replacing epithelial cells as an anti-inflammatory modulator for intestinal myeloid populations³.

A second epithelial signaling molecule recently linked to IBD is Hedgehog⁶. Both Indian (Ihh) and Sonic (Shh) Hedgehog are secreted by the intestinal epithelium and signal in a paracrine manner to the mesenchyme⁷. Ihh and Shh are involved in a number of developmental patterning events in the intestine⁸⁻¹⁰ and are also expressed in the adult GI tract⁷. Among the target cells that respond to Hh signals in the adult colon are dendritic cells and macrophages⁶. Interestingly, a non-synonymous SNP in GLI1, a downstream transcription factor and direct target of Hh signaling¹¹, is associated with IBD in three large European populations; the variant GLI1 protein shows reduced transactivation activity in transfected cells⁶. In accordance with the idea that reduced Hh signals might predispose to inflammation, Gli1^{+/-} mice exhibit increased susceptibility to dextran sodium sulfate with markedly upregulated IL23/IL17 signaling⁶. These data suggest the novel hypothesis that the adult intestinal epithelium might use Hh ligands to modulate the tolerogenic response of the lamina propria, but the potential genetic targets of Hh signaling involved in this regulation have not been identified.

The objectives of the current investigation were two fold: 1) to understand how a chronic reduction in Hh signals would impact intestinal health and 2) to learn more about the target genes and pathways that are altered by acute changes in Hh signaling in the intestine. To accomplish the first goal, we carried out a long-term analysis of a bi-transgenic mouse model of Hhip (Hedgehog interacting protein) overexpression, 12.4KVil-Cre × 12.4KVil-flox-LacZ-flox-Hhip (which we call VFHhip). This model, which is described in detail elsewhere (Zacharias *et al.*, submitted), allows analysis of the phenotypes arising from chronic reduction in Hh levels, as Hhip is a pan-Hh inhibitor¹² and VFHhip animals show clear downregulation of the Hh pathway by one month of life. Early phenotypic changes in the VFHhip model include crypt expansion followed by reduction in villus smooth muscle (Zacharias *et al.*, submitted).

Here, we show that older VFHhip animals exhibit villus atrophy and develop spontaneous small intestinal inflammation.

To learn more about the inflammatory pathways acutely regulated by epithelial Hh proteins, we performed microarray analyses on small intestinal mesenchyme samples (from wild type mice) to which purified Hh ligands were added. The results of this microarray indicate that intestinal Hh signaling is primarily pro-myogenic and anti-inflammatory. Further, we show that when intestinal mesenchyme is cultured for 48 hours without epithelium (the Hh source), pro-inflammatory genes are upregulated. Re-addition of Hh ligand alone results in robust down-regulation of the same genes. Thus, we propose that epithelial Hh ligands are important homeostatic modulators of small intestinal tolerance and suggest that chronically reduced Hh signaling may promote intestinal inflammatory disease.

Materials and Methods

Microarray and Bioinformatic analysis

E18.5 intestinal mesenchyme was isolated, cultured and treated with Hh ligand as previously described⁸. RNA preparation, microarray analysis and bioinformatics processing were performed as described in Supplemental Methods. Comparisons used to identify differentially expressed genes are shown schematically in Supplemental Figure 1. Array data are available in the GEO database

(<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hjmxnwmqyuckjc&acc=GSE17840>).

Analysis of VFHhip animals

The generation of VFHhip animals (12.4KVil-flox-LacZ-flox-Hhip $\times$ 12.4KVil-Cre bitransgenic mice) is described elsewhere (Zacharias *et al.*, submitted). Briefly, a 12.4Kb genomic fragment containing the upstream region of the mouse villin gene¹³ was linked to a truncated version of the Hhip cDNA which lacks the transmembrane region⁸. A floxed LacZ cassette was placed between the villin regulatory sequence and the Hhip cDNA; Cre activity (driven by 12.4KVil-Cre¹³) results in the loss of the LacZ cassette, activation of the Hhip cDNA and intestine-specific inhibition of Hh signaling. Tissue processing, immunostaining and functional analysis of isolated myeloid cells were performed as described in Supplemental Methods.

Results

Chronic Inhibition of adult intestinal Hh signals leads to villus loss and spontaneous inflammation

Because recent data suggest that a 50% reduction in Hh signals leads to inflammation of the adult colon⁶, we wished to examine the long-term effects of moderate Hh inhibition in the adult intestine. In VFHhip bi-transgenic mice, Cre expression leads to activation of the pan-Hh inhibitor, Hhip. Hhip expression begins perinatally, several days later than in the previously described 12.4KVilHhip transgenic founders⁸. Q-PCR evaluation of Hh pathway activity in these mice shows that by one month of age, *Gli1* expression is reduced to approximately 30% of normal expression, indicating significant pathway inhibition (Zacharias *et al.*, submitted). Over the first 3 months of life, VFHhip mice exhibit expansion of the proliferative crypt region of the epithelium, mislocalization of intestinal subepithelial myofibroblasts and progressive loss of villus smooth muscle (Zacharias *et al.*, submitted).

Here, we further aged VFHhip animals and examined them for intestinal alterations over a period of 6-10 months. At 6 months of age, all VFHhip animals exhibited deep crypts and areas

of patchy villus loss (Figure 1A-D). Regions devoid of villi were often complicated by inflammation in the lamina propria (Figure 1C,D). Villus smooth muscle was dramatically reduced, as shown by loss of cells positive for both α SMA and desmin (Figure 1E,F). However, there was no evidence of weight loss or wasting at this time.

Between 6 and 10 months of age, areas of villus loss were interspersed with regions exhibiting villus blunting, crypt hyperplasia, mucous cell expansion, and lamina propria inflammation (Figure 2AD). These lesions bore resemblance to those found in human celiac disease; however, no increase in intra-epithelial lymphocytes was seen in inflamed areas. Inflammation was prominent in the lamina propria and with age, was seen more frequently in submucosal regions (Figure 2D). Lesions of variable size and severity were identified throughout the length of the small intestine. The extent, severity and timing of inflammation was somewhat variable but appeared to be progressive; a time-course of disease progression is provided in Supplemental Figure 2A. The colon in these mice exhibited little phenotype, likely because the villin promoter, which drives expression of the Hhip transgene, is much stronger in the small intestine than in the colon¹³. The level of Hhip in the colon of these mice may not be sufficient to inhibit Hh signals.

Interestingly, VFHhip animals developed dermatitis more frequently than single transgenic littermates. By 10 months of age (or at the time of death), 8/10 VFHhip animals had developed visible dermatitis, whereas only 4/15 co-housed single transgenic littermates showed dermatitis (Supplemental Figure 2A). Because IgA deposition is a complication of a characteristic dermatitis in human celiac disease¹⁴, we examined the skin lesions in 5 VFHhip animals and 5 single transgenic littermates for IgA deposits. IgA was observed in the inflamed skin of 3/5 VFHhip animals but not in any of the single transgenic littermates (Figure 2E,F).

Older VFHhip animals stochastically developed diarrhea and rapid weight loss, culminating in death. By 10 months of age, the entire cohort of bi-transgenic VFHhip animals (N=10) had died (Supplemental Figure 2B) while all single transgenic littermates housed with VFHhip animals remained alive (N=14). Since animals with these late stage changes also showed signs of malabsorption (weight loss, diarrhea, lethargy), we assessed the differentiation status of the remaining epithelium. Immunostaining with alkaline phosphatase, a marker of differentiated epithelial cells, revealed a striking lack of differentiated enterocytes (Figure 2G,H), providing potential insight into the clinical wasting displayed by VFHhip animals. Together, these data indicate that long term inhibition of intestinal Hh signaling is associated with crypt hyperproliferation, progressive villus loss and severe inflammatory disease with phenotypic similarities to human inflammatory conditions.

Hh Treatment inhibits expression of inflammatory genes in isolated mesenchyme

To address whether the development of inflammatory disease in older VFHhip mice is due to direct modulation of inflammatory pathways by Hh signals or whether progressive tissue changes (e.g. barrier breaks during villus atrophy) predisposed to inflammatory disease, we examined the acute transcriptional response of intestinal mesenchyme to the addition of Hh ligand. It is known that the epithelium is the primary source of Hh signals throughout fetal and adult life and these Hh signals impact paracrine targets in the mesenchyme, including myofibroblasts, smooth muscle precursors, and differentiated smooth muscle cells⁷. We previously showed that whole intestinal mesenchyme from E18.5 mice can be cleanly isolated from the epithelium⁸. Culture of this isolated mesenchymal fraction for 48 hours in the absence of epithelium results in loss of Hh signal transduction as measured by expression of the direct target gene and Hh transcription factor *Gli1*; Hh signal transduction can be robustly reactivated within the mesenchyme 24 hours after addition of recombinant Shh or Ihh ligand to the mesenchymal cultures (Zacharias *et al.*, submitted). Thus, isolated cultured mesenchyme is an appropriate model to identify genes and pathways that are acutely modulated by Hh signaling.

Isolated intestinal mesenchyme was cultured for 48 hours without passage, and then exposed to recombinant Shh or Ihh ligand (or vehicle) for 24 hours. We identified probeset expression values that were modulated at least 1.5 fold (and $p \leq 0.05$) by Hh treatment. Unexpectedly, we found more downregulated than upregulated probesets with both Shh and Ihh treatment (Supplemental Tables 1 and 2). There was considerable overlap in probesets regulated by Shh and Ihh: 27 common upregulated probesets, including the known targets, *Ptc1* and *Gli1* and 75 common downregulated probesets (Supplemental Tables 3 and 4), further validating the genes associated with these probesets as likely downstream targets (either direct or indirect) of Hh signaling.

We carefully examined the array data for evidence of a differential response to Shh vs. Ihh. Twenty-five potential differential targets with a fold difference of greater than 1.2 and $p \leq 0.05$ were identified; we utilized a low cutoff of 1.2 fold in order to stringently assess differentially regulated targets. However, each of these targets was regulated with both Shh and Ihh treatment in the same direction though with subtle expression differences (data not shown); these differences may therefore be a result of recombinant ligand activity *in vitro* rather than true differential activation by Shh and Ihh ligand. Thus, we found no solid evidence for a differential response of intestinal mesenchyme to Shh vs. Ihh in these studies.

We proceeded to examine those targets that were significantly regulated (≥ 1.5 fold) by both Shh and Ihh in cultured mesenchyme (Supplemental Tables 3 and 4). First, we compared the frequency of most highly-represented Gene Ontology (GO) Biological Process terms in the group of probesets upregulated by both Hh ligands with the frequency of these same terms in the list of probesets expressed in whole mesenchyme (Figure 3A). Second, we utilized DAVID Functional Annotation to identify major pathways modulated in Hh-treated mesenchyme (Table 1). Both analyses revealed that genes upregulated by Hh are strongly associated with developmental processes. MeSH anatomy filtration was used to examine the cell type specificity of upregulated probesets. Six of the 10 top cell types linked to these probesets were muscle-related (Supplemental Table 5), consistent with previous studies that show roles for Hh in intestinal smooth muscle development⁸⁻¹⁰.

Analysis of probesets downregulated by both Shh and Ihh signaling revealed a striking result: these probesets were strongly associated with genes that exhibit pro-inflammatory function (Figure 3B). Major intestinal inflammatory players such as *Il-1 β* , *Il-6*, and several key CC and CXC class chemokines are downregulated by Hh signaling (Supplemental Table 4). In addition, several genes important in the maturation and function of myeloid immune lineages, including *CD11b/Itgam* and *CD14*, are downregulated in response to Hh signals. DAVID functional annotation of these genes suggests that Hh signaling modulates genes associated with immune system process and stimulus response (Table 2). Indeed, MeSH anatomy filtration reveals that the majority of genes regulated by Hh are expressed in myeloid and other immune cell types, bolstering the conclusion that the activation of Hh signaling in intestinal mesenchyme regulates inflammatory processes in these cells (Supplemental Table 6).

Culture of intestinal mesenchyme results in activation of inflammatory pathways

In order to better contextualize the gene expression changes in isolated mesenchyme induced by Hh ligands, we sought to profile the changes associated with the transition of mesenchyme to culture. We compared gene expression in freshly isolated E18.5 mesenchyme¹⁵ to cultured mesenchyme treated with vehicle (the control set for the Hh treatment analysis above); this vehicle-treated mesenchyme had been in culture for a total of 72 hours at the time of analysis. Over 7000 probesets showed changed greater than 2 fold during transition to culture. The vast majority of these genes (~5500) exhibited changes between 2 and 3 fold; we therefore chose a cutoff of ≥ 5 fold to identify those regulated processes that are mostly strongly modified by the transition to culture. This comparison identified 358 upregulated probesets and 76

downregulated probesets (Supplemental Table 7) with expression changes greater than 5 fold in cultured versus uncultured mesenchyme.

Importantly, *Gli1* (−7.3 fold), *Ptch1* (−4.3 fold), and *Hhip* (−12.5 fold), all direct early target genes of Hh signaling^{11,12,16}, were each robustly downregulated in cultured mesenchyme, providing further evidence that removal of epithelium reduces the activity of the Hh pathway (Supplemental Table 7). DAVID Functional Annotation analysis revealed that the major upregulated gene categories during entry to culture include: inflammatory response, cytokine signaling and positive regulation of growth and proliferation (Figure 3C and Supplemental Table 8). Major downregulated processes include muscle and extracellular matrix related-pathways (Supplemental Table 9).

Hh ligands are sufficient to blunt the inflammatory response present in intestinal mesenchyme following removal of the intestinal epithelium and entry to culture

Removal of the epithelium and culture of the mesenchyme is likely to modulate more than just Hh signaling in the mesenchyme, since the epithelium is the source of a number of additional signals that can modify mesenchymal cell response¹⁵. We therefore examined the extent to which activating Hh signaling alone could alter the direction of pathway regulation (i.e. can Hh ligand cause a gene that was downregulated during transition to culture to instead be upregulated?).

First, we identified those probesets that were both downregulated by transition to culture (and removal of the source of Hh ligand) and upregulated by the addition of both Shh and Ihh. This cohort of genes (Supplemental Table 10) includes the known direct Hh targets *Ptch1* and *Gli1*, as well as a recently identified direct target *Myocd* (Zacharias *et al.*, submitted). These findings suggest that this group of genes is likely to contain other novel direct Hh targets. Functional analysis with DAVID indicates that the major process related to this group of probesets is organ development (Supplemental Table 11).

Next, we identified those probesets that were both upregulated by transition to culture and downregulated by the addition of Hh ligand (Supplemental Table 12). Hierarchical clustering identified two clusters of probesets that are expressed at low levels in freshly isolated mesenchyme, are upregulated during the transition to culture, and are downregulated by addition of Hh ligand (Figure 4A). These probesets are associated with genes that are clearly pro-inflammatory (Table 3 and Figure 4B); several CC and CXC cytokines are strongly upregulated by transition to culture and also markedly downregulated by addition of Hh ligand. Additional genes on this list include *IL-1b*, *IL-6*, TLR2, and cell surface markers expressed on myeloid cells including *CD14* and *CD11b*. These data strongly suggest that a) epithelial Hh acts as an anti-inflammatory signal, b) removal of the epithelium has a pro-inflammatory effect on the mesenchyme, and c) addition of Hh ligand alone can largely substitute for the anti-inflammatory activity provided by the epithelium.

Hh signals act directly on CD11b⁺ myeloid cells to blunt the release of IL-6

The MeSH data indicate a clear association of Hh downregulated genes with expression in myeloid cell lineages (Supplemental Table 6). Myeloid cells of other organs, including the colon⁶, respond directly to Hh signals, but small intestinal myeloid lineages have unique characteristics^{3,17,18}, and we wished to assess whether these cells also responded to Hh signals. To this end, we utilized *Gli1*^{+LacZ} animals, which express β -galactosidase under the control of the *Gli1* locus. Because *Gli1* is a direct Hh target expressed only in cells that respond to Hh signaling¹⁹, β -galactosidase expression is a sensitive readout of Hh response in these animals. We examined β -galactosidase expression in small intestinal CD3⁺ T cells, CD19⁺ B cells and in CD11b⁺ and CD11c⁺ myeloid cells. To carefully identify only those cells which expressed

both β -galactosidase and immune markers, we used confocal microscopy to evaluate serial 0.3 μ m optical sections. While CD3⁺ T cells and CD19⁺ B cells do not appear to respond to Hh signals under homeostatic conditions (Figure 5A,B), some, but not all CD11b⁺ and CD11c⁺ myeloid cells respond directly to Hh signals (Figure 5C,D). These results were further confirmed using intestinal mesenchyme isolated from Gli1^{LacZ/+} mice (Figure 5E,F). When these cells were stained with antibodies to CD11b, all cells with a dendritic-like morphology were strongly CD11b⁺ and LacZ⁺ (white arrows, Figure 5E,F). In contrast, CD11b⁺ cells with a larger, more spread morphology were only weakly positive for LacZ (dotted circle, Figure 5E,F).

Finally, to examine the functional effect of Hh ligands on CD11b⁺ cells, we isolated these cells from small intestinal lamina propria, exposed them to Ihh or Shh in culture, and measured the release of the proinflammatory cytokine, IL-6. Treatment with both Hh ligands severely blunted the release of IL-6 (Figure 6). Myeloid lineages are known to be immunomodulatory in the small intestine³ and these findings, in combination with our VFHhip and microarray data, suggest that Hh signals may act to promote the maturation or function of tolerogenic myeloid populations in the small intestine.

Discussion

These data support the hypothesis that Hh signaling is an important epithelial modulator of inflammatory signaling in the small intestinal lamina propria. The transcriptional response of isolated E18.5 mesenchyme to both Shh and Ihh revealed immune response genes and inflammatory pathways among the top downregulated processes. Moreover, exogenous Hh ligand alone can reduce the strongly pro-inflammatory response observed during culture of isolated mesenchyme, indicating that epithelial Hh plays a major role in modifying inflammatory pathways. In accordance with this hypothesis, analysis of adult VFHhip animals demonstrates that chronic reduction of Hh signals in the adult intestine leads to villus loss, spontaneous inflammation, and death. Taken together, these data provide the first direct evidence implicating epithelial Hh signals in modulation of inflammation in the small intestine, and provide insight into the role of this developmental signaling pathway in immune homeostasis in the gut.

One important trend emerging from the microarray experiments is the observation that Hh signals stimulate the expression of genes involved in the development and function of smooth muscle cells, including *Myocd*, *Igf1*, and *Fgfr2*. This is an important observation in concert with extensive data indicating that Hh signals are crucial in smooth muscle development in the GI tract⁸⁻¹⁰, lung²⁰, bladder²¹, ureter²², and vasculature²³. In addition, recent data from our group indicate that Hh signals stimulate development of villus and muscularis mucosa smooth muscle through direct activation of the SRF co-activator, Myocardin (Zacharias *et al.*, submitted). The fact that Myocardin and several other smooth muscle-related genes were also identified in our microarray experiments emphasizes the importance of Hh signals in the specification of smooth muscle cells.

The other major trend of our microarray studies was the unexpected identification of Hh ligands as key regulators of immune pathways in the intestine. Given the role of Hh in fate determination in multiple systems, it is tempting to speculate that the expression changes that we interpret as modification of inflammatory signaling actually represent modulation of the fate or activity of myofibroblast or myeloid cell lineages. Modulation of myeloid cell fate or phenotype would explain why addition of Hh, typically a ligand that promotes gene activation, down-regulates so many pro-inflammatory genes. The major pathways identified in our expression data are clearly associated with myeloid cell innate immunity, and our data demonstrate that myeloid cells respond directly to Hh signaling in the small intestine. Myeloid

cells are key determinants of inflammation in the intestine³, exerting regulatory control and communicating with other cell types to maintain proper immune homeostasis. Differential populations of myeloid cells are pro- or anti-inflammatory and these phenotypes can be modulated during response to external signals⁴. One intriguing possibility is that Hh signals help to create or maintain a proper balance of tolerogenic versus pro-inflammatory myeloid populations in the small intestine; this possibility will need to be formally explored in future studies. Additionally, myofibroblasts have been shown to secrete inflammatory mediators²⁴, and are responsive to Hh signaling throughout life⁷. Reduction in Hh signaling in late gestation⁸ or postnatally (Zacharias *et al.*, submitted) leads to mislocalization of myofibroblasts. Such changes in localization or function stimulated by reduced Hh may provoke altered inflammatory signaling from this regulatory population. Together, the myeloid and myofibroblast populations provide the best candidates for the cellular targets that receive Hh signals intended to regulate immune response and inflammation.

Recent studies in the colon suggest that reduction in Hh signal transduction predisposes to inflammation in both mouse and human⁶. Our results here emphasize the point that functional Hh signals are required to maintain a tolerogenic milieu in the context of the mammalian small intestine. The stochastic nature of the development of significant inflammatory disease in VFHhip suggests that an unknown stimulus is needed to begin the inflammatory process. This stimulus may be the aberrant response to normal trauma in the absence of functional Hh signaling, a barrier breach due to villus loss, or another trigger. Regardless of the precise etiology, Hh signaling is clearly required to protect from such an event as single transgenic littermates of VFHhip animals survive without inflammatory disease.

Hh has now been implicated in both colonic⁶ and small intestinal inflammatory regulation. This is intriguing in light of significant recent studies demonstrating that some human susceptibility loci are associated with celiac disease as well as Ulcerative Colitis (e.g., polymorphism in the IL2/IL21 region of the genome)²⁵. Strikingly, the inflammatory phenotype seen in VFHhip animals shares phenotypic similarities with both human Crohn's disease and celiac disease. The inflammation is patchy and can be transmural, characteristics similar to Crohn's. VFHhip animals also exhibit villus atrophy, crypt hyperplasia, and profound inflammation, mirroring a celiac-like phenotype. Moreover, the wasting disease experienced by older VFHhip animals may be the result of malnutrition secondary to lost absorptive surface after villus loss and inflammation; reduced absorptive surface is a hallmark of celiac disease in humans. Additionally, the prevalence of dermatitis in VFHhip animals mirrors the high incidence of dermatitis herpetiformis in human celiac disease patients¹⁴. While the dermatitis in VFHhip animals may be a result of malabsorption or malnutrition, some VFHhip skin lesions demonstrate IgA deposition, a key finding in celiac-related dermatitis in humans. Finally, loss of smooth muscle may be a first step in the development of inflammation in VFHhip animals. Likewise, in humans with celiac disease, smooth muscle populations are affected; anti-smooth muscle antibodies are often found in celiac patients and may help identify a subset of those patients who are particularly susceptible to advanced disease²⁶.

Our analysis of small intestinal immune cells that respond to Hh signaling indicates that CD11b⁺ cells respond *in vivo* and *in vitro* to Hh ligand. Sorted populations of CD11b cells are functionally capable of responding to Hh signals by down-regulation of IL-6 protein and mRNA. Interestingly, there appear to be at least two different morphological subsets of these cells that demonstrate different levels of Gli expression. The Gli1^{High} expressing cells have the morphology of dendritic cells. Thymic dendritic cells have also been shown to express Gli1 and respond functionally to Hh²⁷. Interestingly, although early studies indicated that T cells are also Hh responding cells^{28,29,30}, ablation of Hh signaling by knockout of *Smoothed* in DN4 T cells has no effect on the differentiation or expansion of CD4 or CD8 populations³⁰.

Our finding that small intestinal T cells do not express Gli1 are in accord with these latter functional studies.

Overall, the data presented here provide novel evidence that Hh signaling is an important anti-inflammatory signal in the small intestine. The inflammatory milieu in the small intestine is specialized, and many studies have shown that tolerogenic signals from both the stroma and epithelium are critical in modulating the tolerogenic response of the small intestine innate immune system^{31,32}. The emerging role of the Hh signaling pathway as important modulator of inflammation identifies an additional cellular signaling molecule from the epithelium as an important factor in balancing the inflammatory response of the mesenchyme. Hh signaling may cooperate with other intestinal tolerogenic signals (e.g., TSLP) to pattern a proper intestinal inflammatory response, but these microarray studies show that Hh alone can dramatically alter inflammatory signaling in isolated mesenchyme. Reduction in this homeostatic Hh influence may predispose to a disordered inflammatory response even in the presence of an otherwise normal immune system and may contribute to human gastrointestinal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Chronic inhibition of Hh signaling leads to villus atrophy

Studies were done in VFHhip double transgenic mice and their single transgenic (ST) littermates (carrying either 12.4KVil-Cre or 12.4KVil-flox-LacZ-flox-Hhip transgene). A) Villus structure is normal in 6 month old ST mice. B) Six month old VFHhip animals exhibit villus loss with dramatic crypt expansion. C,D) Regions of villus loss and inflammation in 6 month old VFHhip mice. E, F) αSMA/desmin double staining for visualization of myoblasts (green), myofibroblasts (red), and differentiated smooth muscle (yellow). VFHhip animals exhibit extensive loss of villus smooth muscle by 6 months of age.

Figure 2. VFHhip animals develop spontaneous small intestinal inflammation

A-D) H&E staining of ST (A) and VFHhip (B-D) mice at 8-10 months of age. B) VFHhip animals exhibit inflammatory cells in the lamina propria beneath blunted villi. C) Some focal areas of villus blunting, crypt expansion and goblet cell hyperplasia resemble the histology of human celiac disease. D) In focal areas, inflammation extends submucosally. E,F) Staining for IgA in skin lesions of ST and VFHhip mice. VFHhip mice frequently show dermatitis (Supplemental Figure 2B) and 60% of these lesions contain depositions of IgA (F), while skin lesions observed in ST animals (E) do not. G,H) In ST, alkaline phosphatase staining is prominent on villus tips (G), while blunted villi in VFHhip animals exhibit loss of functional absorptive surface (H).

Figure 3. Ihh and Shh regulate developmental and inflammatory pathways in isolated intestinal mesenchyme

Overrepresented Biological Processes were identified using DAVID for A) genes upregulated by Hh, B) genes downregulated by Hh, and C) genes upregulated by transition to culture. The percentage of genes associated with each of the 20 most overrepresented processes (identified by p value, most significant at the top, blue bars) were compared to the percentage of genes associated with the same process in the list of all mesenchymally-enriched genes (citation 26, red bar).

Figure 4. Hh ligand reverses up-regulation of pro-inflammatory genes that occurs in isolated mesenchyme

A) K mean clustering analysis of gene expression from freshly isolated mesenchyme (Mes), cultured mesenchyme (Veh), and Hh ligand-treated cultured mesenchyme (Shh). Gene expression level is indicated by color, with blue indicating low expression, black moderate expression, and yellow high expression. Two clusters of genes that were low in freshly isolated mesenchyme, more highly expressed in cultured mesenchyme, and reduced with Hh ligand addition were visualized. B) Genes that are both upregulated by culture and downregulated by Hh are overwhelmingly associated with inflammation and immune response.

Figure 5. Small intestine myeloid lineages respond directly to Hh signaling

Co-staining in Gli1^{+/LacZ} animals for nuclear LacZ and CD3 (A), CD11b (B), CD11c (C) and CD19 (D). Nuclei detected with anti-LacZ antibody are shown in red, and immune lineages are in green. Images shown are representative 0.3μm confocal slices taken at 600x. Some CD11b⁺ and CD11c⁺ myeloid cells have LacZ positive nuclei (arrows) while CD3⁺ and CD19⁺ lymphoid cells do not. The section in (D) is from the edge of a lymphoid nodule. E,F) Staining of isolated intestinal mesenchymal cells in culture with anti-LacZ (red) and anti-CD11b (green). Overlay of red, green and blue (DAPI) channels (E) and analysis of red channel alone (F) shows that CD11b⁺ cells with stellate morphology (arrows) exhibit robust staining for LacZ. A second population of CD11b⁺ cells with spread morphology (dotted circle) has lower nuclear levels of Gli1. Additional Gli1 positive cells are visible, likely myofibroblasts.

Figure 6. Hedgehog signaling inhibits intestinal lamina propria leukocyte IL-6 production

Lamina propria mononuclear cells (LPMC) or sorted CD11b+ cells of C57BL/6 mice were isolated from the small intestine as described in Supplemental Methods. Cells were maintained in culture for 3 hours then treated with Shh (250ng/mL), Ihh (250ng/mL), or medium for 48 hrs. The level of IL-6 was measured by ELISA. Both Hh ligands inhibited the production of IL-6 by these two cell populations. *** $P < 0.001$.

Table 1

DAVID Functional Annotation Clusters for Genes Regulated by Shh and Ihh

Functional Cluster	Enrichment Score	Top Term	Top Term FE	Top Term P Value	Top Term FDR
Processes Associated with Genes Upregulated by Shh and Ihh Treatment of Intestinal Mesenchyme					
1	2.78	GO:0035295 Tube Development	15.52	2.28E-04	4.35E-01
2	1.6	INTERPRO: IPR013783 Immunoglobulin-Like Fold	6.29	1.92E-03	3.66E+00
3	1.24	GO:0030098 Cytokine Activity	17.5	1.16E-02	2.00E+01
4	1.19	UP_SEQ_FEATURE Disulfide Bond	2.37	1.80E-02	3.19E+01
5	1.17	GO:0048513 Carbohydrate Binding	4.41	1.41E-04	2.69E-01
6	1.13	SP_PIR_KEYWORD Transmembrane Protein	7.57	1.34E-02	1.89E+01
7	0.89	GO:0044249 Cellular Biosynthetic Process	3.17	6.06E-02	6.97E+01
8	0.78	GO:0048598 Embryonic Morphogenesis	12.8	3.18E-03	5.90E+00
9	0.75	GO:0044238 Primary Metabolic Process	1.39	7.52E-02	7.76E+01
10	0.64	GO:0006955 Immune Response	4.51	5.15E-02	6.36E+01
Processes Associated with Genes Downregulated by Shh and Ihh Treatment of Intestinal Mesenchyme					
1	12.22	GO:0009605 Response To External Stimulus	10.52	5.31E-17	1.01E-13
2	10.81	GO:0002376 Immune System Process	7.4	3.36E-17	6.43E-14
3	8.98	GO:0005125 Cytokine Activity	15.54	6.26E-14	1.11E-10
4	3.54	GO:0042221 Response To Chemical Stimulus	7.15	3.20E-09	6.12E-06
5	2.3	GO:0030246 Carbohydrate Binding	5.96	3.50E-04	6.22E-01
6	1.78	GO:0007159 Leukocyte Adhesion	61.89	9.95E-04	1.88E+00
7	1.74	GO:0008201 Heparin Binding	10.86	3.04E-02	4.23E+01
8	1.67	GO:0009967 Positive Regulation Of Signal Transduction	12.84	9.52E-05	1.82E-01
9	1.54	KEGG_PATHWAY Toll-Like Receptor Signaling Pathway	6.67	1.58E-03	1.96E+00
10	1.44	SP_PIR_KEYWORD Innate Immunity	13.44	2.05E-02	2.74E+01

DAVID Functional Annotation Clusters for Genes Upregulated by Culture and Downregulated by Hh Ligand

Table 2

Functional Cluster	Enrichment Score	Top Term	Top Term FE	Top Term P Value	Top Term FDR
1	8.89	GO:0008009	79.64	6.62E-17	2.00E-13
2	1.88	KEGG_PATHWAY	8.89	3.84E-04	4.78E-01
3	1.88	SP_PIR_KEYWORD	9.69	1.64E-03	2.52E+00
4	1.62	GO:0043123	21.60	8.16E-03	1.45E+01
5	1.53	GO:0004871	1.75	1.08E-02	1.76E+01
6	0.98	GO:0009310	14.16	1.83E-02	2.97E+01