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Interleukin-1 β Promotes Gastric Atrophy Through Suppression of Sonic Hedgehog

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Abstract

Background & Aims—In both human subjects and rodent models, *Helicobacter* infection leads to a decrease in Shh expression in the stomach. Sonic Hedgehog (Shh) is highly expressed in the gastric corpus and its loss correlates with gastric atrophy. Therefore, we tested the hypothesis that proinflammatory cytokines induce gastric atrophy by inhibiting Shh expression.

Methods—Shh-LacZ reporter mice were infected with *H. felis* for 3 and 8 weeks. Changes in Shh expression were monitored using β -galactosidase staining and immunohistochemistry. Gastric acidity was measured after infection and IL-1 β was quantified by qRT-PCR. Mice were injected with either IL-1 β or omeprazole prior to measuring Shh mRNA expression and acid secretion. Organ cultures of gastric glands from wild type or IL-1R1 null mice were treated with IL-1 β then Shh expression was measured using qRT-PCR. Primary canine parietal or mucous cells were treated with IL-1 β . Shh protein was determined by immunoblot analysis. Changes in intracellular calcium were measured by Fura-2.

Results—All major cell lineages of the corpus including surface pit, mucous neck, zymogenic and parietal cells expressed Shh. *Helicobacter* infection reduced gastric acidity and inhibited Shh expression in parietal cells by 3 weeks. IL-1 β produced during *Helicobacter* infection inhibited gastric acid, intracellular calcium and Shh expression through the IL-1 receptor. Suppression of

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parietal cell Shh expression by IL-1 β and omeprazole was additive. IL-1 β did not suppress Shh expression in primary gastric mucous cells.

Conclusion—IL-1 β suppresses Shh gene expression in parietal cells by inhibiting acid secretion and subsequently the release of intracellular calcium.

INTRODUCTION

Helicobacter-induced gastritis in the corpus leads to hypochlorhydria, and oxyntic gland atrophy, a lesion that predisposes the stomach to cancer ^{1,2}. However, the mechanism by which chronic inflammation triggers loss of the parietal cells atrophy is not understood. Atrophy is marked by the loss of normal gastric glands then replacement with either gastric (pseudopyloric/spasmolytic peptide-expressing) or intestinal metaplasia ³⁻⁶. Thus, there is a shift in the cellular composition of the stomach from oxyntopeptic lineages to mucous lineages.

Shh has recently been implicated as a critical factor in gastric organogenesis and glandular differentiation ^{7,8}. Prior analysis of Shh null mice suggested that intestinal metaplasia (IM) develops in the stomach ⁹. However a more recent study of the Shh null mice revealed that stomach exhibits features of hyperplasia rather than IM since the stomach exhibited gastric markers ¹⁰. Nevertheless, both studies demonstrate that the gastric mucosa is abnormal in the absence of Shh. Several studies have documented the expression of Shh in the gastric corpus ^{7,8,11-13}. However there is conflicting information regarding which cell types actually express Shh. In early studies, Shh protein expression in the mouse stomach was observed in the mucous neck, parietal and chief cells ^{7,8}. However, Fukaya et al. reported expression only in the parietal cells ¹². Employing in-situ hybridization and immunostaining techniques, El-Zaatari et al. reported that Shh mRNA and protein expression is located in surface pit and parietal cells ¹¹. Subsequently, Minegishi et al. reported that Shh mRNA and protein is expressed in the basal glandular region including parietal and chief cells ¹³. To date, no genetic models have been used to study the expression of Shh in the adult stomach. We used the Shh-LacZ reporter mice to study the cellular origin of Shh expression in the stomach and its response to inflammation in vivo.

Polymorphisms in the pro-inflammatory cytokine IL-1 β promoter correlate with an increase in IL-1 β gene expression, gastric atrophy and cancer in *Helicobacter*-infected subjects ¹⁴. Recently, transgenic overexpression of human IL-1 β in the stomach was shown to induce dysplasia and gastric cancer ¹⁵. Apart from its role in inflammation, IL-1 β is also a potent inhibitor of gastric acid secretion ¹⁶. Prior studies have suggested a role for gastric acid in regulating Shh expression ^{13,17-19}. Therefore, we determined if IL-1 β induces gastric atrophy through its ability to suppress Shh gene expression.

METHODS

Mice

Shh-LacZ mice have been described elsewhere ²⁰. This line carries the β -galactosidase cDNA inserted into the 3' UTR of the Shh locus. The mice were maintained as a homozygous colony. All mice were fasted overnight with free access to water before analysis. The study was performed with the approval of the University of Michigan Animal Care and Use Committee, which maintains an American Association for Assessment and Accreditation of Laboratory Animal Care facility.

β -Galactosidase (β -gal) staining

The stomach was opened along the greater curvature and the gastric contents were washed in ice cold PBS. The stomach was fixed in fresh 4% paraformaldehyde/PBS (pH 7.0-7.5) for 1h

at 4°C, then rinsed three times for 30 min with β -gal rinse buffer (100 mM sodium phosphate pH 7.3, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40) at room temperature. The tissue was incubated for 16 h at 37°C in β -gal staining solution (β -gal rinse buffer, 25 mg/ml X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide). The tissue was rinsed with β -gal rinse buffer for 30 min at room temperature, and post-fixed overnight in 4% paraformaldehyde/PBS. The tissue was processed and paraffin-embedded prior to sectioning.

mRNA Analysis

Resected tissue was collected in Trizol reagent (Invitrogen) total RNA was extracted, purified, and DNase-treated using the RNeasy kit (Qiagen). Using the iScript cDNA synthesis kit (BioRad), cDNA was synthesized from 1 μ g of total RNA. QRT-PCR was performed using the BioRad I cycler with SYBR Green dye (Molecular Probes). Each 20 μ l reaction contained 2 μ l of reverse-transcribed product, 1x PCR buffer, MgCl₂, 100 nM of each primer, 1x SYBR Green, 10 nM fluorescein, 200 mM dNTPs, and 0.025 U of Platinum Taq polymerase (Invitrogen). Each PCR amplification was performed in triplicate wells with the following conditions: 3 min at 95°C, 40 cycles of 9 s at 95°C and 1 min at 60°C, followed by 1 min at 55°C. Melt curve analysis was used to assess the purity of the product. Beacon software (BioRad) was used to design the mouse primer sequences, Shh reverse 5'-ATCGTTCGGAGTTTCTTGAT-3', Shh forward 5'-ATGTTTCTGGTGATCCTTGCT-3', GAPDH reverse 5'-TATTATGGGGTCTGGGATGG-3', GAPDH forward 5'-TCAAGAAGGTGGTGAAGCAGG-3' and TaqMan primers (Applied Biosystems) for IL-1 β and GAPDH were used. Data for each gene were normalized to the expression of GAPDH.

Immunoblot analysis

Protein was loaded on a 4--20% SDS-PAGE gradient gel for immunoblot analysis. The membranes (Hybond-C Extra Nitrocellulose, Amersham Biosciences) were blocked with Detector Block (KPL, Gaithersburg, MD) for 1 h at room temperature followed by an overnight incubation with a 1:200 dilution of the goat polyclonal anti-Shh antibody (Santa Cruz Biotechnology, sc-1194), or a 1 h incubation with 1:5000 GAPDH (Chemicon) antibody. The membranes were washed twice for 10 min each in TBS-0.1% Triton-X (TBST) and incubated for 1 h with a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated secondary anti-goat, or anti-mouse antibodies. The membranes were washed five times for 30 min with TBST. Proteins were visualized using enhanced chemiluminescence (Lumilight substrate, Roche Applied Science, Mannheim, Germany).

Immunohistochemical Staining

X-gal stained tissue sections were deparaffinized and rehydrated. The sections were then washed with 1x PBS, blocked with 20% normal goat or donkey serum and incubated with a 1:50 dilution of HRP conjugated anti-GSII, a 1:500 dilution of rabbit anti-Intrinsic factor (David Alpers, Washington University) or a 1:125 dilution of mouse anti-H⁺,K⁺ ATPase β subunit (Medical & Biological Laboratories Co., LTD) for 1 h. Staining was visualized with avidin-biotin complexes by using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine for the substrate (DAB, DAKO). H⁺, K⁺ ATPase staining was performed using the Ark-labeling kit (Dako). Morphometric analysis was performed on LacZ positive sections for Shh by counting a total of 200 epithelial cells from each of five different high power fields per section per mouse. The results were expressed as percent of Shh positive cells. Morphometric analysis for parietal cells was performed by counting a total of H⁺,K⁺ ATPase positive cells and both H⁺,K⁺ ATPase and LacZ positive cells from 5 well-oriented glands in random fields for each mouse section. The number of LacZ

positive parietal cells was expressed as a percent of the total number of H, K-ATPase positive cells per mouse. Warthin-Starry silver staining was performed to document persistent bacterial infection even 8 weeks after inoculation by identifying the distinctive spiral *Helicobacter* organisms (Microbiology Labs, Michigan State University).

Immunofluorescence

Immunofluorescence staining was performed on 5 μ m paraffin sections. Tissue sections were deparaffinized, rehydrated and blocked with 20% normal goat or donkey serum. A 1:100 dilution of goat anti-Shh (Santa Cruz), 1:500 dilution of rabbit anti-UEA1 (Sigma Aldrich), a 1:800 dilution of mouse anti-H⁺,K⁺-ATPase- β subunit (Medical & Biological Laboratories Co., LTD) and anti-IL-1R1 (BD Pharmingen) antibodies were used on X-gal stained sections to identify specific cell types followed by a 1:500 dilution of fluorophore-labelled Alexafluor secondary antibody (Molecular probes). Nuclei were counterstained with DAPI and images were generated on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) using a SpotCD camera.

Bacterial Strain and Culture Conditions and Mouse infection

Helicobacter felis (*H. felis*, ATCC, Manassas) was grown on Trypticase Soy Agar with 5% Sheep Blood, plates (BD Diagnostics BBL). Broth cultures were made by inoculating the bacteria in Brucella broth. Mice were gavaged three times over 3 days with 10^8 *H. felis* organisms in 100 μ l of *brucella* broth. Control mice were gavaged with *brucella* broth alone.

Organ culture

Stomachs from 8 weeks old mice were opened along the greater curvature, and washed in ice cold PBS then twice in ice cold 10% fetal bovine serum (FBS) supplemented RPMI before incubating in 6ml of 10% FBS-RPMI with either vehicle (PBS) or IL-1 β (7.5ng/ml) for 3h.

Primary Canine Cell Preparation and Culture

Canine parietal and mucous cells were isolated using the modified elutriation method 21–24. The isolated parietal cells and mucous cells were cultured in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) containing 0.1 mg/ml gentamicin, 50 units/ml penicillin G, 0.01 mg/ml ciprofloxacin and 2% DMSO (Sigma) on a 35 mm dish coated with 150 μ l of growth factor reduced Matrigel (BD Biosciences). The cells were treated with either vehicle (PBS), 100 ng/ml IFN- γ or 100 ng IL-1 β for 6 h and whole cell extracts were prepared using RIPA buffer and analyzed by western blot.

Gastric Acidity

The mice were starved overnight. After euthanizing, the stomach was cut along the greater curvature and rinsed with 2 ml of 0.9% NaCl solution. Residual gastric debris was removed by centrifugation at 3,000 rpm for 5 min, and the supernatant was collected to determine acidity by titration using 0.005N NaOH. The concentration of gastric acid was expressed as μ Eq H⁺.

IL-1 β Treatment

Ten week old wild type C57BL/6 mice were injected intraperitoneally with IL-1 β (125 ng in 100 μ l/mouse) or vehicle (PBS). The mice were euthanized 3 h after receiving IL-1 β .

Omeprazole Treatment

Ten week old wild type mice were injected intraperitoneally with 10 μ mol omeprazole. The stock solution of omeprazole (80 μ mol/ml) was dissolved in vehicle dimethyl sulfoxide/

polyethylene glycol (4.5/0.5 vol/vol) and stored at -20°C until use. All mice were euthanized 2 h after receiving omeprazole or vehicle.

Measurements of Intracellular Calicum

See Supplemental Methods Section.

Statistical Analysis

The significance of the results was tested using the unpaired t test and one-way ANOVA using commercially available software (Graphpad Prism, GraphPad Software, San Diego, CA). A p value < 0.05 was considered significant.

RESULTS

Both mucous and oxyntic cell lineages express Shh

Results from in situ hybridization and immunohistochemical analyses to determine the cellular origin of Shh expression in the stomach have been inconclusive. The recent development of Shh-LacZ reporter mice²⁰ provided us with a novel tool to study the expression of Shh in vivo. Whole mounts of stomach tissue from non-transgenic (WT) and transgenic Shh-LacZ reporter mice were incubated with β -galactosidase substrate (X-gal). We observed that Shh expression was highest in the corpus, low in the antrum and undetectable in the forestomach (Fig. 1B). Also there was modest Shh expression in Brunner's glands, though some background LacZ staining was observed in the non-transgenic stomach stained simultaneously (Fig. 1A). Since the LacZ gene was inserted into the 3' UTR of the mouse Shh locus, quantitative analysis of Shh expression was performed on tissue extracts from the corpuses of wild type and Shh-LacZ reporter mice to rule out disruption of the endogenous locus. Similar levels of Shh mRNA and protein were found in both the WT and Shh-LacZ reporter mice demonstrating that the reporter gene did not alter endogenous Shh expression (Fig. 1C, 1D). Next, we demonstrated that all major cell types in the corpus express Shh (Fig. 1E) by colocalizing LacZ expression with surface pit (UEA1), mucous neck (GSII), parietal (H^+ , K^+ ATPase) and chief (intrinsic factor) cell markers (Fig. 2A–D). Of note, parietal cells primarily at the base of the oxyntic gland expressed little to none of the LacZ reporter suggesting a gradient of Shh expression in parietal cells. The corpus glands closest to the forestomach consistently exhibited the highest level of Shh expression (Fig. 1E). Stromal, and smooth muscle cells were completely devoid of Shh expression.

Helicobacter infection promotes rapid loss of Shh expression

Although *Helicobacter* infection in both human subjects and rodent models leads to decreased Shh expression, the mechanism regulating Shh expression has not been examined. Therefore, we infected the Shh-LacZ reporter mice with *H. felis* for three and eight weeks to generate chronic gastritis. Shh expression was reduced at both three weeks and eight weeks of *H. felis* infection (Fig. 3A–D). Bacterial infection was confirmed by Warthin-Starry silver staining of the X-gal stained paraffin sections from the infected mice (Suppl. Fig. 1A, C). At three weeks of infection, the loss of Shh expression was patchy with a slight decrease in acid levels that became significant after 8 weeks of infection (Suppl. Fig. 1B,D), but without evidence of significant parietal cell loss (Fig. 3E,F). By eight weeks of infection, the loss of Shh was uniformly distributed throughout the mucosa and the degree of Shh expression was significantly greater than the decrease in parietal cells numbers (Fig. 3G, H). Reduced Shh expression was more prominent at the base of the oxyntic glands with surface pit cells still retaining a substantial amount of Shh expression (Fig. 3D). The changes in Shh expression between uninfected and infected mice were quantified using morphometric analysis (Fig. 3F, H). Moreover, the loss of Shh expression preceded physical loss (atrophy) of the parietal cells

(Fig. 3B, D, E,G). To demonstrate that loss of Shh expression preceded parietal cell atrophy, we co-stained sections from the Shh-LacZ mice with antibodies to the parietal cell marker H^+ , K^+ ATPase and quantified the number of co-staining cells by morphometry. Indeed by 8 weeks of *Helicobacter* infection, there was a significant decrease in the number of parietal cells that were LacZ positive compared to the degree of reduction in parietal cell numbers (Fig. 4; Fig. 3E,G).

IL-1 β inhibits Shh expression

It is well established that IL-1 β inhibits parietal cell acid secretion¹⁶. Moreover, deletion of the H^+ , K^+ ATPase α or β subunit gene locus results in profound hypochlorhydria, atrophy, and alters gastric epithelial cell differentiation^{25, 26}. Prior studies have suggested a role for gastric acid in regulating the expression of Shh¹⁷. Therefore, we examined if *Helicobacter*-induced parietal cell inhibition of Shh is mediated by IL-1 β . A time course analysis of IL-1 β mRNA expression after three and eight weeks of *Helicobacter* infection was performed. There was a 3- and 5-fold increase in IL-1 β mRNA expression respectively (Fig. 5A, B). Consistent with the increase in the levels of IL-1 β , there was a loss of gastric acid production, with significant reduction by eight weeks of *Helicobacter* infection (see Fig. 4J). To assess if IL-1 β alone mimicked the effect of *Helicobacter* infection on Shh expression, mice were injected with IL-1 β . We first demonstrated that IL-1 β indeed inhibits gastric acid (Fig. 5C). Next, mice were injected with omeprazole, an established inhibitor of gastric acid and specifically the H^+ , K^+ ATPase enzyme, either alone or a combination of IL-1 β (Fig. 5C). We noted that a combination of omeprazole and IL-1 β did not suppress gastric acid further than treatment with omeprazole alone. Injection of IL-1 β alone effectively suppressed Shh expression by ~60% (Fig. 5D). Interestingly, omeprazole recapitulated the inhibitory effect observed with IL-1 β alone (Fig. 5D). However, there was an additive repressive effect on Shh gene expression when the mice received both IL-1 β and omeprazole (Fig. 5D). To determine if the differences observed were due to changes in the number of parietal cells, we performed morphometric analysis on gastric tissue sections from vehicle and mice treated for 3h with IL-1 β (Suppl. Fig. 1). Although there was no statistically significant difference in the number of parietal cells, confocal analysis of the immunostained glands was revealed morphologic differences with both IL-1 β and omeprazole treatments consistent with inhibition of acid secretion (Suppl. Fig. 1A–D). We concluded from these results that although both IL-1 β and omeprazole inhibit gastric acid secretion that their effect on Shh gene expression proceeds through parallel and therefore additive pathways. This result is consistent with the knowledge that omeprazole inhibits acid secretion by directly binding to and inactivating the H^+ , K^+ -ATPase enzyme. By contrast, the effect of IL-1 β works through a receptor-mediated mechanism.

IL-1R1 is required for inhibition of Shh by IL-1 β

To confirm that the effect of IL-1 β on Shh gene expression was mediated through the IL-1 β receptor, we first established the location of the IL-1 β receptor in the gastric mucosa. Tissue sections from wild type mice were co-stained with anti-IL-1R1 antibody and the parietal cell marker H^+ , K^+ -ATPase. Both mucous and parietal cell lineages expressed the IL-1R1 receptor (Fig. 6A, B). Next, to test if IL-1 β signaled through the IL-1R1 receptor to inhibit Shh expression, organ cultures from the stomachs of wild type or IL-1R1 null mice were treated with IL-1 β for 3 h. IL-1 β suppressed Shh mRNA expression in wild type stomach cultures, but not when the organ cultures were prepared from IL-1R1 null mice (Fig. 6C). Therefore IL-1 β inhibits Shh gene expression through activation of the IL-1 β receptor.

IL-1 β inhibits parietal cell specific Shh expression

Helicobacter infection induced preferential loss of Shh expression in the oxyntic glandular region with surface pit cells still retaining a substantial amount of Shh expression (Fig. 3D).

Therefore, we tested if IL-1 β mimics *Helicobacter* infection by differentially regulating Shh expression in primary parietal versus mucous cell cultures. Primary canine parietal cells were treated with two proinflammatory cytokines IFN γ or IL-1 β for 6 h and protein was prepared for Western blot. Although IFN γ significantly induced Shh expression, IL-1 β treatment dramatically inhibited Shh gene expression in primary parietal cells (Fig. 7A). By contrast, IL-1 β treatment had no effect on Shh expression in primary mucous cell cultures (Fig. 7B). These results correlated well with our in vivo observations showing rapid loss of Shh expression from the parietal cells, but retained expression in the surface pit mucous cells.

IL-1 β inhibits release of intracellular calcium in parietal cells

It has been shown previously that blocking parietal cell acid secretion also inhibits the release of intracellular calcium²⁷. Therefore, we tested the possibility that IL-1 β inhibits acid secretion by modulating intracellular calcium. The ratio-metric calcium binding dye Fura 2 is membrane-permeable, is taken up by the parietal cells and changes its emission wavelength when bound to calcium. It is thus used to follow changes in intracellular calcium²⁸. Indeed, perfusing primary parietal cells with a pH of 7 reduced the intracellular calcium signal determined by the Fura 2 dye (Fig. 8A). By contrast, perfusing the primary cells with an acidic pH of 5 dramatically increased the Fura 2 signal. However, perfusing the cells with a pH of 5 and IL-1 β prevented the expected increase in intracellular calcium. Fig. 8B shows a representative tracing in real time showing the dramatic increase in intracellular calcium perfusing with pH 5 buffered the an rapid decrease in intracellular calcium. Thus, we concluded that IL-1 β interferes with gastric acid mediated release of intracellular calcium.

DISCUSSION

The molecular details of how chronic inflammation in the corpus leads to atrophy are not well understood. Shh has been implicated as a critical factor in gastric gland organogenesis and differentiation⁷. Several studies have shown in both human subjects and rodent models that *Helicobacter* infection leads to a decrease in Shh expression. However, no studies have examined why Shh expression in the stomach is inhibited by inflammation and more specifically have examined a role for pro-inflammatory cytokines in mediating the suppression. Thus the goal of this study was to understand how a specific pro-inflammatory cytokine leads to loss of Shh expression.

Using a LacZ reporter mouse, we identified the gastric cell types expressing Shh exclusive of other hedgehog family members e.g., Ihh and Dhh. We noted that the corpus glands near the junction with the forestomach, and the surface pit cells were stained most intensely with the X-gal substrate. There appeared to be two gradients of expression in the glandular stomach proceeding from anterior (corpus) to the reduced levels in the posterior (antrum) stomach; and highest at the gastric lumen (surface pit) then diminishing towards the gland base. There was a gradient of Shh expression in the parietal cells that varied along the vertical axis with the highest expression in neck zone that diminished in the parietal cells at the gland base. The parietal cells at the base of the glands express less H⁺,K⁺-ATPase and are destined for destruction²⁹. Thus, one explanation for differences in detection of Shh expression by different authors might be due to differences in the regions of the stomach examined. For example, a prior study reported an absence of Shh expression in the antrum⁷. By contrast, we detected patchy X-gal staining in the antral gland primarily in surface pit cells (data not shown). Thus the overall expression in the gastric antrum was not as robust as that observed in the corpus and might have been easily overlooked.

We observed a rapid loss in Shh expression with *Helicobacter* infection. Although patchy, the loss of Shh expression did not correlate with the presence of bacteria. It was not known if the loss of Shh expression during *Helicobacter* infection was due to the physical loss of Shh-

expressing cells or to the inhibition of Shh expression. Our results here would support the argument that Shh expression occurs prior to the physical loss of parietal cells observed with long-standing *Helicobacter* infection. Moreover, the persistent expression of Shh in the surface pit cells after *Helicobacter* suppressed expression in the parietal cells suggests cell specific differences in the response of the various gastric cell types to inflammation.

IL-1 β inhibits gastric acid secretion both in vivo in mice and in vitro, in primary parietal cell cultures from rabbit and dog ^{24, 30–32}. In the current study, inhibition of Shh gene expression upon IL-1 β treatment was demonstrated both in vitro and in vivo using three different model systems. First, we showed that injection of the mice with IL-1 β suppresses Shh expression in vivo. Second, IL-1 β treatment of gastric organ cultures inhibited Shh expression. Third, IL-1 β treatment of primary parietal cells inhibited Shh protein expression. In addition, we found that injection of the proton pump inhibitor omeprazole was also a potent inhibitor of Shh expression. Collectively, inhibition of acid secretion from chronic *Helicobacter* infection, IL-1 β or omeprazole treatment suppressed Shh expression.

The effect of IL-1 β on Shh is a hitherto unknown role for this cytokine and requires activation of the IL-1 receptor. Since Stepan et al. reported that Shh induces H⁺,K⁺-ATPase gene expression ³³, it is likely that chronically suppressed levels of Shh will eventually reduce enzyme levels and subsequently acid production. Moreover since the lack of the H⁺,K⁺-ATPase enzyme is sufficient to induce gastric atrophy ^{25, 26}, inhibiting Shh expression in parietal cells is likely to promote atrophy. We have shown previously that low acid conditions in both mice and human gastric mucosa is sufficient to prevent Shh processing ¹⁹. Taken together with the effect on Shh gene expression, IL-1 β initiates self-perpetuating, hypochlorhydric conditions in the stomach in which Shh is neither expressed nor processed to its biologically active form.

An important finding was that IL-1 β effectively blocks the release of intracellular calcium. Indeed, a prior study reported the IL-1 β blocks carbachol-induced acid secretion by blocking an increase in intracellular calcium and subsequently calcium-dependent protein kinase C ²⁸. Another study suggests that PKC activation regulates Shh gene expression ³⁴. The dependence of Shh expression on intracellular calcium levels would explain why IL-1 β inhibits Shh. Further IL-1 β synergizes with direct inhibition of acid production by omeprazole since an alkaline pH also inhibits intracellular calcium. Therefore both treatments inhibit acid and the release of intracellular calcium but through different mechanisms. Moreover, the effect of gastrin, histamine and cholinergic agonists on the parietal cell also work through calcium-dependent mechanisms.

Clinically, it is known that hypochlorhydria predisposes to gastric atrophy. While this outcome is generally assumed to be due to loss of parietal cells, our results would suggest that hypochlorhydria occurs before there is actual loss of parietal cell mass. Rather, the cells become inactive in the presence of specific pro-inflammatory cytokines unable to release calcium from intracellular stores, which subsequently reduces Shh gene expression. Once Shh is lost due to reduced transcription and processing, parietal cells are lost (atrophy) then are replaced by mucous cells (metaplasia). Thus, an important conclusion revealed by this study is that effective inhibition of gastric acid by a pro-inflammatory cytokine is a link that furthers our understanding of how chronic inflammation can induce parietal cell atrophy by suppression of Shh.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Shh is predominantly expressed in the corpus

Whole mounts of stomachs from a nontransgenic (NTg) (A) and a Shh-LacZ reporter mouse (B) are shown after incubating the tissue in a β-galactosidase substrate (X-gal) for 16h. Quantitative RT-PCR was performed on corpus RNA from nontransgenic and Shh LacZ reporter mice. Shown is the ratio of Shh to GAPDH mRNA (C). The mean ± SEM for three mice is shown. Immunoblots of protein isolated from the corpus of nontransgenic and Shh-LacZ mice are shown in D, the immunoblot for Shh was re-blotted for GAPDH. Protein expression was quantified using the image-J software (NIH). The mean ± SEM for Shh/GAPDH is shown (D). Gastric tissue sections extending from the proximal portion of the stomach, the forestomach (black arrow) into the corpus is shown (E). Nuclear β-galactosidase activity was detected in the epithelial cells (X-gal and haematoxylin-eosin staining).

Figure 2. Both mucous and oxyntic cell lineages express Shh

Whole mounts of stomachs from Shh-LacZ reporter mice were incubated in the β -galactosidase substrate (X-gal) for 16h prior to paraffin embedding. The stomach was sectioned then immunostained for cell specific markers. Ulex europaeus (UEA1) for pit cells (A), Griffonia simplicifolia (GSII) for mucous neck cells (B), H^+-K^+ -ATPase (HK) for parietal cells (C), and intrinsic factor (IF) for chief cells (D). Magnification is 400x; insets are 1000x.

Figure 3. *Helicobacter* infection suppresses Shh expression

X-gal and haematoxylin-eosin staining of three months old Shh LacZ uninfected (A) and mice infected with *H. felis* for three weeks (B) and four months old uninfected (C) and mice infected with *H. felis* for eight weeks (D). Magnification is 200x and the insets are 100x magnification. The number of H⁺,K⁺-ATPase+ cells per gland at 3 and 8 weeks of infection is shown (E,G). Shh-LacZ positive cells after three weeks (F) and eight weeks of *H. felis* infection were analyzed by morphometry (H). Shown is the mean $\pm$ SEM for LacZ positive cells from six mice per group for three weeks and ten mice per group for eight weeks *, P < 0.05 relative to uninfected mice. NS = not significant.

Figure 4. Loss of Shh expression precedes parietal cell atrophy

Immunohistochemical staining of parietal cells on X-gal stained paraffin sections from uninfected (A) and eight weeks *H. felis* infected mice (B). Magnification is 200x and 1000x for the insets. (C) Morphometric analysis of total number of parietal cells from uninfected and eight weeks *H. felis*-infected mice. Changes in the parietal cells expressing Shh (X-gal) were analyzed for uninfected and eight week-infected *H. felis* mice. Shown is the mean $\pm$ SEM for fifteen glands from three mice per group *, $P < 0.05$ relative to uninfected mice.

Figure 5. IL-1β inhibits Shh expression

Quantitative RT-PCR was performed on total stomach RNA from uninfected and Shh LacZ reporter mice infected with *H. felis* for three weeks (A) (n=6 uninfected, n=4 infected), or eight weeks (B) (n=10). Shown is the relative induction of IL-1β mRNA normalized to GAPDH. The mean ± SEM is shown *, p<0.05 compared to uninfected mice. Acid secretion from mice injected with vehicle (open bar), IL-1β (black bar), omeprazole (grey bar) and omeprazole and IL-1β (open bar) was measured expressed as μEq of acid H⁺ (C). Shown is the mean ± SEM. *, P<0.05 relative to uninfected or vehicle treated mice. Quantitative RT-PCR for Shh was performed on total corpus RNA from vehicle (open), IL-1β (filled bar), omeprazole (grey) and omeprazole and IL-1β-treated (open bar) mouse stomachs. Shown is the ratio of Shh mRNA to GAPDH mRNA (D). The mean ± SEM for eight mice is shown. *, P<0.05 compared with vehicle treated mice. **P<0.05 OM compared to IL-1β plus OM treatment. NS, not statistically significant.

Figure 6. IL-1R1 is required for inhibition of Shh by IL-1β

Co-localization of H, K-ATPase (green) (A) and IL-1β receptor1 (red) (B). (C) Quantitative RT-PCR was performed on RNA extracted from organ cultures isolated from gastric corpus of PBS-treated (open bars) or IL-1β-treated (black bars) stomachs isolated from WT or IL-1R1KO mice and treated with PBS (grey bar) or IL-1β (black bar). Shown is the ratio of Shh to GAPDH mRNA expressed as the mean $\pm$ SEM for four mice. *P < 0.05 compared with vehicle treated mice. NS, not statistically significant.

Figure 7. IL-1 β inhibits parietal cell specific Shh expression

Primary cultures of canine parietal cells were treated with PBS, IFN γ and IL-1 β . Whole cell lysates were analyzed by immunoblot (A). Primary cultures of canine mucous cells were treated with PBS and IL-1 β . Whole cell lysates were analyzed by immunoblot (B). Shown is the mean $\pm$ SEM for three separate parietal cell and mucous cell preparations. *P< 0.05 compared with PBS treatment. NS, not statistically significant.

Figure 8. IL-1 β inhibits acid-dependent release of intracellular calcium

Primary cultures of canine parietal cells pre-loaded with the Fura 2-AM dye were perfused with A) HBSS at pH 7 (upper panel), pH 5 (middle panel), or pH 5 with IL-1 β (lower panel). B) Shown is a representative measurement of calcium binding to Fura 2 performed three times.