Requirement of Notch activation during regeneration of the intestinal epithelia

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Okamoto R, Tsuchiya K, Nemoto Y, Akiyama J, Nakamura T, Kanai T, Watanabe M. Requirement of Notch activation during regeneration of the intestinal epithelia. Am J Physiol Gastrointest Liver Physiol 296: G23–G35, 2009. First published November 20, 2008; doi:10.1152/ajpgi.90225.2008.—Notch signaling regulates cell differentiation and proliferation, contributing to the maintenance of diverse tissues including the intestinal epithelia. However, its role in tissue regeneration is less understood. Here, we show that Notch signaling is activated in a greater number of intestinal epithelial cells in the inflamed mucosa of colitis. Inhibition of Notch activation in vivo using a γ-secretase inhibitor resulted in a severe exacerbation of the colitis attributable to the loss of the regenerative response within the epithelial layer. Activation of Notch supported epithelial regeneration by suppressing goblet cell differentiation, but it also promoted cell proliferation, as shown in in vivo and in vitro studies. By utilizing tetracycline-dependent gene expression and microarray analysis, we identified a novel group of genes that are regulated downstream of Notch1 within intestinal epithelial cells, including PLA2G2A, an antimicrobial peptide secreted by Paneth cells. Finally, we show that these functions of activated Notch1 are present in the mucosa of ulcerative colitis, mediating cell proliferation, goblet cell depletion, and ectopic expression of PLA2G2A, thereby contributing to the regeneration of the damaged epithelia. This study showed the critical involvement of Notch signaling during intestinal tissue regeneration, regulating differentiation, proliferation, and antimicrobial response of the epithelial cells. Thus Notch signaling is a key intracellular molecular pathway for the proper reconstruction of the intestinal epithelia.

The intestinal epithelia are composed of four lineages of intestinal epithelial cells (IECs) that arise from intestinal stem cells (1). Recent studies have shown that various signals such as Wnt, Sonic hedgehog, and bone morphogenetic protein interact within the stem and progenitor cells of the intestinal epithelia to finely regulate the expansion and the cell fate decision of IECs. Other studies have revealed that Notch signaling may also play critical roles in the maintenance of the intestinal epithelia (20).

Notch signaling is a signaling pathway known to regulate differentiation and proliferation of cells in diverse adult tissues (1). Activation of Notch receptor is mediated by the cleavage of its intracellular domain (NICD), and this intracellular domain translocates from the cell membrane to the nucleus, thereby functioning as a transcriptional activator of target genes such as Hes1 (10, 25). The functional role of Notch signaling in the intestine was first described in a study of Hes1-null mice; depletion of Hes1 was associated with significant increases in the secretory lineage IECs (9). Other studies have shown that the activation of Notch promoted proliferation of crypt progenitor cells and directed their cell fates toward absorptive but not secretory lineage cells (6, 28, 33). A recent study suggested that Notch might also function in postmitotic IECs, directing their cell fates toward secretory lineage cells (42). Thus these studies have suggested that Notch signaling functions in the intestine to regulate differentiation and proliferation of IECs, contributing to the maintenance and the homeostasis of the intestinal mucosa. However, the role of Notch signaling in tissue regeneration is less understood.

Damage of the intestinal epithelia is observed in a wide variety of diseases, such as acute intestinal infections, radiation injuries, or idiopathic inflammatory bowel diseases (23). Once the epithelial layer is damaged, it responds by restoring the continuity and integrated structure via activating the stepwise regeneration program (16). The initial response is called restitution, which is the redistribution of remaining IECs to rapidly cover the damaged area. This initial step is usually completed in an extremely short period of time and thus does not require the proliferation or expansion of IECs (19). However, in the next step, the rapid expansion of IECs is necessary to rebuild the proper structure of the epithelia. This response is manifested by the appearance of the regenerative epithelia in the intestine, showing a marked expansion of the proliferating compartment consisting of undifferentiated IECs. However, the exact molecular mechanisms involved in this critical step of intestinal epithelial regeneration has never been described.

Another change that is observed in the intestine during such a regenerative process is the ectopic expression of antimicrobial peptides by IECs. Paneth cells usually secrete peptides such as lysozymes, α-defensins, or PLA2G2A, and this helps to maintain the ideal environment for the stem and progenitor IECs within the small intestinal crypts. The ectopic expressions of these antimicrobial peptides by IECs are frequently observed in the inflamed colonic mucosa (5, 8), and such expressions likely support the local immune system in providing an ideal environment for the regeneration of the damaged mucosa.

In this study, we show that Notch signaling is activated in many IECs in the inflamed mucosa of murine colitis. Results show that the activation of Notch is critical for the proper regeneration program in the epithelial layer and that it helps to suppress goblet cell differentiation and promote cell proliferation. A comprehensive analysis identified a novel group of genes regulated by Notch in IECs, which included

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a gene encoding an antimicrobial peptide called PLA2G2A. Such functions of Notch activation were present not only in the mice intestine but also in the human intestine. Finally, the clinical relevance of Notch-mediated regeneration is analyzed in ulcerative colitis (UC). Thus Notch signaling is a key-signaling pathway involved in intestinal tissue regeneration, in fine regulation of differentiation and proliferation, and in antimicrobial activities in IECs. Our findings point to a novel molecular target for agents that could promptly regenerate the intestinal mucosa in a wide range of intestinal diseases.

MATERIALS AND METHODS

Mice. C57BL/6J mice at 8 wk of age were purchased from Japan Clea. Mice were housed and maintained in the animal facility of Tokyo Medical and Dental University. The institutional animal use and care committee approved the study.

In vivo experiments. Induction of colitis was performed as previously described (17). Briefly, mice were fed ad libitum with 1.75% dextran sodium sulfate (DSS, Bio Research of Yokohama) for 5 consecutive days, followed by distilled water for another 5 days. For inhibition of Notch activation, mice were orally administered with either 5% DMSO (vehicle, VEC) or LY411,575 (LY) (10 mg/kg) dissolved in 0.5% (wt/vol) methylcellulose (WAKO), once daily for 5 consecutive days. Twenty-four mice were separated into four groups: 1) fed distilled water for 5 days followed by daily administration of vehicle alone (VEC, n = 6) for 5 days, 2) fed distilled water for 5 days followed by daily administration of LY411,575 (LY, n = 6) for 5 days, 3) fed 1.75% DSS for 5 days followed by daily administration of vehicle (DSS + VEC, n = 6) for 5 days, and 4) fed 1.75% DSS for 5 days followed by daily administration of LY411,575 (DSS + LY, n = 6) for 5 days. The whole body weight of mice was measured everyday. They were euthanized 12 h after the final administration. Colonic tissues were subjected to hematoxylin and eosin staining and analyzed by histological scoring following the criteria described elsewhere (21). Flow cytometry of thymocytes and splenocytes were performed as previously described (35, 41).

Immunoblot analysis. Immunoblots were performed as described elsewhere (18). The primary antibodies used were anti-Cleaved Notch1 (1:1,000, Cell Signaling Technology), anti-Hes1 (1:4,000, a kind gift from Dr. T. Sudo), and anti-β-actin (1:5,000, Sigma). Proteins were visualized either by the ECL Advance Western Blotting Kit (GE Healthcare) or ECL Western Blotting Kit (GE Healthcare).

Cell culture. The cell cultures and transfections of plasmid DNA were performed as described elsewhere (18). The inhibition of Notch signaling was achieved by the addition of LY411,575 (1 μM), synthesized according to Wu et al. (38). A cell line expressing Notch1 intracellular domain (Tet-On NICD1 cells) under the control of tetracycline or doxycycline (DOX, 100 ng/ml, Clontech) was generated as described elsewhere (18), using LS174T cells as parent cells. The cell lines were supplemented with Blasticidin (7.5 μg/ml, Invitrogen) and Zeocin (750 μg/ml, Invitrogen) for their maintenance.

RT-PCR assays. RT-PCR was performed as described elsewhere (18). Quantitative analyses using the SYBR green master mix (Qiagen) was performed by ABI 7500 (Applied Biosystems). Primer sequences for human β-actin, G3PDH, or MUC2 have been previously described (30). The primer sequences for other genes are summarized in Table 1. The results are shown as the means of the data collected from two rounds of assays, with each assay performed in triplicate. The data were statistically analyzed with paired Student’s t-tests.

Human intestinal tissue specimens. Human tissue specimens were obtained from patients who underwent surgery for the treatment of Crohn’s disease, UC, or colon cancer at Yokohama Municipal General Hospital or Tokyo Medical and Dental University Hospital. Written informed consent was obtained from each patient, and the study was approved by the ethics committee of Yokohama Municipal General Hospital and Tokyo Medical and Dental University.

Immunohistochemistry. Immunohistochemistry using intestinal tissue specimens has been described elsewhere (12). The same antibodies used in immunoblot analysis were also used for the immunohistological staining of NICD1 and Hes1. The other antibodies used were anti-human Ki-67 (1:50, MIB-1, DAKO), anti-human PLA2G2A (1:200, sc-14468, Santa Cruz Biotechnology), anti-human MUC2 (1:100, Ccp58, Santa Cruz Biotechnology), and anti-mouse Ki-67 (1:50, TEC-3, DAKO). Microwave treatment (500 W, 10 min) in 10 mM citrate buffer was required for staining human tissues in Hes1, Ki-67, and NICD1 and for staining mice tissues in Ki-67. The tyramide signal amplification (Molecular Probes) was used for immunofluorescent detection of NICD1. Staining was visualized by an avidin-biotin-peroxidase complex (ABC) elite kit (Vector) using diaminobenzidine as a substrate or by secondary antibodies conjugated with Alexa-594 or Alexa-488 (Molecular Probes). The quantification of Hes1 (Fig. 1B), Alcian blue, Ki-67, or NICD1 (Fig. 8B) was conducted by the examination of nine randomly selected longitudinal sections of crypts selected from at least three different individuals. The data were statistically analyzed with paired Student’s t-tests.

Microarray. Microarray analysis was performed using the Acegene human oligo chip 30K subset A (Hitachi software). Total RNA was collected before and after 24 h of NICD1 expression in LS174T cells and labeled using the Amino Aryl Message Amp aRNA kit (Ambion). The complete dataset of the analysis has been submitted to the NCBI Gene Expression Omnibus (GEO) and is accessible through GEO accession number GSE10136.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>Human Hes1</td>
<td>5′- ATGCCAGCTGATATAATTGGAG -3′</td>
</tr>
<tr>
<td>Human Notch1</td>
<td>5′- CGGAACTAATATACCCCTCT -3′</td>
</tr>
<tr>
<td>Human PLA2G2A</td>
<td>5′- ACCATGAGACCTCTTCTACTG -3′</td>
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<tr>
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<td>5′- TCAACACGAGACCCAAACGAAAG -3′</td>
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<tr>
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</tr>
<tr>
<td>Mouse TNF-α</td>
<td>5′- CTACGGCTGCGTGCCAGGCTCGT -3′</td>
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<tr>
<td>Mouse IFN-γ</td>
<td>5′- GAGATGCTGCTTGCCAGTCCG -3′</td>
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<td>Mouse IL-1a</td>
<td>5′- GGGCGAGATGAGAGATCAGGG -3′</td>
</tr>
<tr>
<td>Mouse IL-6</td>
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</tr>
<tr>
<td>Mouse PLA2G2A</td>
<td>5′- AAGATCGGCAAAAATGCGAAA -3′</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>5′- TCTCTACATGCTGCTGCTGAG -3′</td>
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Table 1. Primers used in the present study
Plasmids. Hes1p-Luc, containing six tandem-repeats of the RBP-Jk binding site, was a kind gift from Dr. Kageyama (Kyoto, Japan). PL2-A-Luc was generated by cloning a 2778-bp sequence of the human PLA2G2A gene (corresponding to nucleotides -2,758 to +20 of the promoter region) into a pGL3 basic vector (Promega). MUC2-Luc (40) was a kind gift from Dr. Yuasa (Tokyo, Japan). Tetracycline-dependent expression of NICD1 was achieved by cloning the gene encoding the intracellular portion of the mouse Notch1 (amino acid 1,704–2,531) into the pcDNA4/TO/myc-his vector (Invitrogen). All constructs were confirmed by DNA sequencing.

Immunostaining of cultured cells. Staining of cultured cells has been previously described (30). Detection of the MUC2 antibody was carried out either by the standard ABC method or by the Alexa 594-conjugated secondary antibody (Molecular Probes). The quantification of cells positive for MUC2 staining was performed by examining six randomly selected fields (three fields each in two independent counts) under ×400 magnification. The data were statistically analyzed with paired Student’s t-tests.

ELISA. For PLA2G2A protein quantification, 1 × 10⁶ cells were cultured in 2 ml of medium with or without DOX and analyzed with the human-PLA2 enzyme immunoassay kit (Cayman Chemicals). The incorporation of BrdU was examined by seeding cells at various cell densities in the 96-well plate, supplemented with DMSO or LY411,575. The BrdU was added 8 h before the end of culture, and the cells were subjected to analysis with the cell proliferation ELISA kit (Roche Diagnostics). The results are shown as the means of data collected by two rounds of assays, with each assay performed in triplicate. The data were statistically analyzed with paired Student’s t-tests.

Reporter assays. The reporter assay was performed as previously described (18). Each assay was performed in triplicate, and the results were normalized using the Renilla luciferase activity. The results are shown as the means of normalized arbitrary units, and the data were statistically analyzed with paired Student’s t-tests.

RESULTS

Hes1 is expressed in crypt epithelial cells of DSS-colitis. Since previous studies have evaluated the contribution of Notch signaling in the maintenance of mice intestinal epithelium (28, 33, 34), we sought to examine the role of Notch signaling in mice colitis. At first, we analyzed the expression of Hes1, a direct target gene of Notch, in mice with colitis induced by the oral administration of DSS (DSS-colitis, Fig. 1A). In the normal colon, crypts are predominantly composed of mature goblet cells that produce mucin. In such crypts, Hes1 is expressed in crypt epithelial cells and an increase in Hes1-positive cells was observed in the inflamed mucosa of DSS-colitis mice. The expressions of both Hes1 and Ki-67 were observed in a larger population of IECs residing at the lowest part of the crypt, which is also where Ki-67-positive IECs are found. In sharp contrast, the clear loss of mucin production was observed in the inflamed mucosa of DSS-colitis mice. The expressions of both Hes1 and Ki-67 were observed in a larger population of IECs, which were distributed from the bottom to the most upper regions of the crypt, suggesting that Notch signaling was activated in these IECs. The quantitative analysis of the immunostaining revealed significant increases in Hes1-positive IECs within the crypts of the DSS-colitis mice (Fig. 1B). These findings suggested that Notch signaling is activated in a greater number of IECs in DSS-colitis, which might be closely related with the greater number of proliferating IECs and the loss of mucin-producing IECs.

LY411,575 inhibits Notch activation and promotes goblet cell differentiation in mice intestine. To further examine the role of Notch signaling in colitis, we used LY411,575, a γ-secretase inhibitor (GSI) that is known to block Notch activation in vivo (14, 27, 36). Oral administration of LY411,575 for 5 consecutive days significantly reduced the expression of...
Hes1 mRNA in mice intestine, suggesting that Notch activation was inhibited (Fig. 2A). In contrast, the expression of MUC2 mRNA was significantly increased by LY411,575, suggesting that the number of goblet cells increased. Consistent with this, histological analysis showed marked increases in mucus-producing IECs in the intestines of the LY411,575-treated mice (Fig. 2C). Consistent with reports from previous studies (27, 36), these results showed that LY411,575 could
simultaneously inhibit Notch activation and promote differentiation to goblet cells in the mice intestine.

We also found marked atrophy of the thymus in LY411,575-treated mice (Supplemental Fig. S1A). Supplemental data for this article are available on the American Journal of Physiology Gastrointestinal and Liver Physiology website. Further analysis of the thymus revealed that the total number of thymocytes was significantly reduced (Supplemental Fig. S1B) and that the tissue architecture was disrupted (Supplemental Fig. S1C).

Analysis of CD4/CD8 expression revealed a significant proportional reduction in double-positive cells (Supplemental Fig. S1D) and a reduction in the absolute number of cells (Supplemental Fig. S1E), suggesting that there was a significant loss of immature cells in the thymus with LY411,575 treatment. However, such an effect of LY411,575 was not present in the spleen (Supplemental Fig. S1, A–E). These findings clearly showed that the LY411,575 treatment had a systemic effect, affecting the thymus in addition to the intestine.

**LY411,575 exacerbates DSS-colitis by impairing epithelial regeneration.** Using the methods described, we designed an experiment to examine the effect of Notch inhibition during colitis (Fig. 2B). Mice were separated into four groups: vehicle alone (VEC), LY411,575 alone (LY), DSS with vehicle (DSS + VEC), and DSS with LY411,575 (DSS + LY). As of day 5, the total body weights showed significant reductions from day 0 in DSS-treated mice (Fig. 2B) compared with the weights of those without DSS (the day when DSS treatment was started is designated as day 0). However, the DSS + LY mice showed even greater reductions in weight as of day 8; their reductions in body weight were significantly greater than the weight reductions in DSS + VEC mice (Fig. 2B). This severe loss of body weight observed in DSS + LY mice was also fatal because two mice in this group died at the time of euthanasia (fatality rate = 2/6, 33.3%). No deaths were observed in any other experimental group. These results suggested that LY411,575 significantly exacerbates the clinical course of DSS-colitis. A histological analysis of LY or DSS + LY mice showed a marked increase in goblet cells in the small intestine, confirming the effect of LY411,575 treatment (Fig. 2C). The increase in goblet cells was also observed in the colon of LY mice. A histological analysis of DSS + VEC mice showed a clear induction of colitis, as shown by the marked increase in inflammatory cells and the elongation of goblet cell depleted crypts. However, in sharp contrast, DSS + LY mice showed a severe loss of the epithelial layer in addition to an infiltration of inflammatory cells, which appeared to lack signs of epithelial regeneration (Fig. 2C). A histological scoring of the colonic tissues revealed increased ulcer formation and epithelial injury in DSS + LY mice compared with DSS + VEC mice, whereas no significant changes were observed in the degree of inflammation (Fig. 2D). Consistent with this, the mRNA expression of proinflammatory cytokines was increased in the colon of DSS-treated mice, but no clear differences were observed between DSS + VEC and DSS + LY mice (Supplemental Fig. S2).

For further analysis, we examined the expression of Hes1 and Ki-67 in the inflamed region of the colonic tissues. An increase in Hes1- or Ki-67-positive IECs was confirmed in DSS + VEC mice (Fig. 2E). However, both Hes1 and Ki-67 expression appeared to be markedly lost in the colonic crypts upon LY411,575 treatment (Fig. 2E). These results indicated that LY411,575 inhibits Notch activation and promotes goblet cell differentiation but also strongly inhibits proliferation of IECs, leading to a poor regenerative response and a severe exacerbation of DSS-colitis.

**LY411,575 promotes goblet cell differentiation but inhibits proliferation of IECs in vitro.** Previous in vivo results suggested that Notch activation might play critical roles in both the differentiation and proliferation of IECs. We further examined the in vitro effect of LY411,575 upon human colonic epithelial cell lines LS174T and HT29. As shown by the immunoblot analysis, the endogenous expression of both NICD1 and Hes1 was completely inhibited within LS174T cells by LY411,575 treatment (Fig. 3A). Consistent with this, RT-PCR analysis showed a marked decrease in Hes1 mRNA expression with LY411,575, which was maintained for up to 72 h (Fig. 3B). These data confirmed that LY411,575 could directly inhibit the activation of Notch within IECs.

Under this condition, we examined whether LY411,575 could promote goblet cell differentiation in vitro. Quantitative RT-PCR analysis showed a significant increase in MUC2 mRNA expression with LY411,575 treatment in both LS174T and HT29 cells (Fig. 3C). Consistent with this, a marked induction of MUC2 protein expression was observed in both of the cell lines that were treated with LY411,575 (Fig. 3D, red signal), resulting in a significant increase in the MUC2-positive cell population (Fig. 3E). The Alcian blue staining also showed a marked increase in mucin-producing cells in both cell lines with LY411,575 (Fig. 3F, black arrow). However, LY411,575 appeared to inhibit the proliferation of both cell lines since the incorporation of BrdU was significantly downregulated by LY411,575 (Fig. 3G). These results collectively showed that LY411,575 could directly inhibit Notch activation in IECs, which might subsequently promote goblet cell differentiation but also inhibit cell proliferation.

**Activation of Notch1 suppresses goblet cell phenotype, but upregulates PLA2G2A secretion in human IECs.** To further analyze the function of Notch activation in IECs, we gen-
erated a subline of LS174T cells (Tet-On NICD1 cells), in which forced expression of NICD1 could be induced in a tetracycline- or DOX-dependent manner. Immunoblot analysis of Tet-On NICD1 cells showed a clear induction of NICD1 and a subsequent increase in Hes1, with DOX addition (Fig. 4A). Consistent with this, the reporter activity of Hes1p-Luc was significantly upregulated with the induction of NICD1 in Tet-On NICD1 cells, indicating that there was an upregulation of the transcriptional activity of the Hes1 gene (Fig. 4B). These results confirmed that Tet-On NICD1 cells could express the functional NICD1 protein with DOX addition.
Using this cell line, we found that the upregulation of NICD1 expression in LS174T cells significantly downregulated MUC2 mRNA expression (Fig. 5A). Further analysis with a microarray identified a group of genes that were up- or downregulated with NICD1 expression (Supplemental Tables 1 and 2). Among these genes, we focused on PLA2G2A, a gene expressed by Paneth cells, as it showed the most significant induction with NICD1 expression. Quantitative RT-PCR confirmed an upregulation of PLA2G2A mRNA expression with the NICD1 expression (Fig. 5A). Consistent with this, although the MUC2 protein expression was markedly suppressed (Fig. 5B), with resulting significant decreases in MUC2-positive cells (Fig. 5C) and mucin-producing cells (Fig. 5D), the PLA2G2A secretion was upregulated with NICD1 expression (Fig. 5E). These changes appeared to be regulated at the transcriptional level since the reporter activities of MUC2-Luc and PLA2-Luc showed a significant decrease and increase, respectively, with NICD1 expression (Fig. 5F). These results showed that, although the activation of Notch1 within LS174T cells suppressed goblet cell phenotype, it also upregulated the secretion of PLA2G2A, suggesting that the activation of Notch1 might surprisingly promote the acquisition of the specific functions of Paneth cells.

Notch1 is activated in crypt epithelial cells of the human intestine. Since we found that Notch signaling might regulate cell proliferation, goblet cell differentiation, and Paneth cell-specific function within IECs, we sought to clarify its relevance in human intestinal diseases. We first examined whether components of the Notch signaling pathway are expressed in the human intestine. An RT-PCR analysis of human intestinal tissues or epithelial cell lines successfully detected mRNAs of both Notch1 and Hes1 (Fig. 6A). The immunohistochemistry for NICD1 and Hes1 revealed that these proteins are expressed in the nuclei of crypt IECs (Fig. 6B). Similar to our observations in mice, the distribution of NICD1-positive or Hes1-positive IECs corresponded to that of Ki-67-positive IECs (Fig. 6B). Also, a magnified view of the staining showed a positive staining of NICD1 in columnar-shaped IECs and Paneth cells (Fig. 6B, black arrow) but not in goblet-shaped IECs (Fig. 6B, red arrowhead). Double staining of MUC2 and NICD1 confirmed the lack of NICD1 expression in goblet cells (Fig. 7A), whereas double staining of PLA2G2A and NICD1 confirmed expression of NICD1 in Paneth cells (Fig. 7B). These results strongly suggested that the NICD1 might function in vivo in the human intestine in a similar manner as was revealed in the in vitro study.

Increased activation of Notch1 is observed in the mucosa of UC. UC is one of the major forms of inflammatory bowel diseases, characterized by the persistent inflammation and ulcer formation in the colon. In the active region of UC, a loss of goblet cells, an ectopic expression of Paneth cell genes, and an increase in IEC proliferation are all known to be common pathological findings (7, 8, 13, 23). Thus our results strongly suggested that all of these pathological findings in UC might be mediated by the activation of Notch1 in IECs. We performed

![Image](http://ajpgi.physiology.org/)
histological analysis and found that in the crypts of UC, mucin production is markedly decreased (Fig. 8A, top, blue), whereas the number of Ki-67-expressing cells are markedly increased, distributing from the bottom to the uppermost part of the crypt (Fig. 8A, bottom, brown). In such crypts, NICD1-expressing cells showed the same distribution as Ki-67-expressing cells (Fig. 8A, middle, brown), suggesting that Notch1 is activated in an expanded proliferating cell population within the crypts of...
A quantitative analysis revealed that the number of IECs expressing NICD1 or Ki-67 per crypt is significantly increased, whereas the number of IECs producing mucin is significantly decreased in the crypts of UC (Fig. 8B). We also looked for IECs expressing PLA2G2A within the colonic crypts. There was no expression of PLA2G2A in the crypts of the normal colon (Fig. 9A). However, an ectopic expression of PLA2G2A was clearly found in the crypts of the colon epithelia with UC (Fig. 9B). Our histological analysis revealed that Notch1 is clearly activated in such IECs ectopically expressing PLA2G2A (Fig. 9, C and D). Such activation of Notch1 in PLA2G2A-expressing cells could also be found in less inflamed regions of UC where there were fewer PLA2G2A-expressing IECs (Fig. 9, E and F).

From these results, we confirmed that Notch1 is activated in a greater number of crypt IECs in UC, presumably mediating goblet cell depletion, cell proliferation, and ectopic expression of PLA2G2A. We suggest that such Notch1-mediated changes...
Fig. 7. Human Notch1 is not activated in IECs expressing MUC2 but is activated in IECs expressing PLA2G2A. A: human Notch1 was not activated in IECs expressing MUC2 in vivo. Double staining for MUC2 (red) and NICD1 (green) using human colonic tissue is shown. NICD1 and MUC2 were expressed in distinct populations of epithelial cells (left, ×400). A magnified view (right, ×1600) clearly shows cytoplasmic staining of MUC2 in goblet-shaped cells (yellow arrow), whereas nuclear staining of NICD1 in columnar-shaped cells (white arrowhead). B: human Notch1 is activated in IECs expressing PLA2G2A in vivo. Double staining for PLA2G2A (red) and NICD1 (green) using a human small intestinal tissue is shown. NICD1 and PLA2G2A were coexpressed in IECs residing at the lowest part of the crypt, suggesting activation of Notch1 in Paneth cells (yellow arrow, original magnification ×1000).

Fig. 8. Increased activation of Notch1 is observed in the crypts of patients with ulcerative colitis (UC). A: decreased expression of mucin and increased expression of both NICD1 and Ki-67 were observed in crypts of patients with UC. Mucin expression was examined by Alcian blue staining, whereas expression of NICD1 or Ki-67 was examined by immunohistochemistry with the use of human colonic tissues. Inner column shows magnified view of the upper (Upper) and lower (Lower) crypt areas identified by dashed line in the outer column. A marked decrease in Alcian blue-positive IECs is observed in a crypt of a patient with UC (top). In contrast, a marked increase in IECs expressing NICD1 (brown, middle) or Ki-67 (brown, bottom) was observed in patients with UC. Distribution of IECs expressing NICD1 or Ki-67 was restricted to the lower part of the crypt in normal colon, but it extended to the most upper region of the crypt in UC (original magnification, outer column ×400, inner column ×1600). B: significant decrease in IECs expressing mucin and significant increase in IECs expressing NICD1 or Ki-67 were observed in crypts of patients with UC. Quantitative analysis of the histological staining for mucin, NICD1, and Ki-67 is shown. Number of IECs positive for Alcian blue staining or immunohistochemical staining for NICD1 and Ki-67, respectively, were counted per crypt and normalized by total number of IECs. Results are shown as percent positive IECs per crypt. Error bars represent SD. *P < 0.05 on the Student’s t-test.
observed in the mucosa of UC are not detrimental changes contributing to the persistence of the disease, but rather they are positive responses that help to regenerate the damaged epithelia, thereby aggressively contributing to the termination and recovery from the disease.

DISCUSSION

To date, several studies using knockout mice have revealed various functions of Notch signaling in IECs; one critical function is that of regulating the cell fates of IECs (31). The recent model accepted in such studies implicates Notch activation as a positive regulator of absorptive cell differentiation but a negative regulator of the differentiation of secretory lineage cells, including goblet cells. However, studies have suggested that Notch activation not only acts to determine the cell fates of progenitor IECs, but it may also regulate the number of proliferating populations within the crypt (6, 28, 33). Our results are consistent with the previous observations, and they further highlight the critical role of Notch activation in a situation when the rapid expansion of IECs is required (e.g., during the regeneration process in UC). Since the in vivo phenotype of Notch inhibition showed not only the loss of absorptive lineage cells but also the loss of the entire epithelial layer, this suggested that the activation of Notch may contribute to the expansion of both absorptive and secretory precursor cells and even stem cells. This is consistent with the observation by Vooijs et al. (34) that IECs that matured into absorptive cells must have also experienced Notch activation during development from the stem cell. Thus our results demonstrated the importance of Notch activation in the expansion of multi-lineage precursor IECs, whose function becomes critically required when tissue damage is present. In contrast, although Notch activation was predominant in the proliferating IECs of the colitic mucosa, its role in postmitotic IECs might be of less importance (42).

A recent study has shown that the chronic inhibition of Notch activation using LY411,575 (for up to 15 consecutive days) could impair the development of lymphoid cells (14, 36). Thus it may be possible that such an effect of LY411,575 might have altered the local immune function of the DSS-treated mice and thereby exacerbated their colitis. Indeed, LY411,575 proved to have a systemic effect, especially on the development of thymocytes (Supplemental Fig. S1). However, no effect was observed on splenocytes (Supplemental Fig. S1). Also, no effect was observed on local production of proinflammatory cytokines (Supplemental Fig. S2). Thus, although it is possible that LY411,575 might have some effect on the inflammatory response, its involvement on the exacerbation of the present colitis model may be minimal.

Also, GSI has been reported to promote the differentiation and inhibit proliferation of mice intestinal adenoma through the inhibition of Notch activation (33). Therefore, GSIs have been reported to have an antitumor effect (32). However, our results showed that the effects of GSIs may not be specific for tumor
cells. GSIs have almost the same effect on progenitor cells of the normal and regenerating crypt, which becomes critically toxic once the epithelia have been damaged. Thus caution is needed with the use of GSIs when intestinal tissue damage is present.

Although studies have revealed various extrinsic factors promoting the regeneration of the intestinal epithelia (2, 3), the intracellular mechanism mediating the regenerative process has not been fully elucidated (15). Our data show that Notch activation maintains the larger number of IECs in the immature state, thereby promoting the proliferation and supporting the rapid recovery of IECs needed to restore proper epithelial structure. Thus we identified Notch signaling as one of the main intracellular pathways mediating the organized regenerative response of the intestinal epithelia. Although we know that several ligands and receptors of the Notch pathway are expressed in the intestine (24, 26), we do not know the precise mechanism by which these ligands activate Notch receptors, in particular IECs. A recent study by Riccio et al. (22) clearly showed that both Notch1 and Notch2 function redundantly in the intestinal epithelia and that they directly regulate the cell cycle progression of crypt progenitor cells. Thus an analysis of the Notch ligand expression is needed to understand the mechanism by which these Notch receptors could be activated during epithelial regeneration and the mechanism by which such activation could be downregulated at the later stage of regeneration.

One of our surprising findings was the upregulation of PLA2G2A in Notch-activated IECs, suggesting that Notch might also modulate immune functions of IECs. PLA2G2A is usually expressed in Paneth cells, and it is known to have an antimicrobial effect (4). The loss of the continuity of the epithelial layer allows various and abundant microorganisms to invade the submucosal area, thereby promoting inflammation and further destruction of the mucosa. Thus the local secretion of PLA2G2A at the damaged mucosal area may be quite beneficial for limiting bacterial invasion and providing a proper environment for regeneration. However, previous reports have shown that PLA2G2A is also expressed by neutrophils and macrophages accumulating at the inflamed mucosa of colitis (29, 39). Consistent with this, we observed an infiltration of PLA2G2A-positive cells in the lamina propria of inflamed mucosa in UC (Fig. 9, C–F). An RT-PCR analysis of DSS-colitis showed a significant upregulation of PLA2G2A expression in the inflamed colonic mucosa (Supplemental Fig. S3). However, such an upregulation was not inhibited with LY411,575 treatment, suggesting that the expression of PLA2G2A by neutrophils or macrophages might be less dependent on Notch activation. In those cells, intracellular pathways such as NF-κB might function to promote expression of PLA2G2A by neutrophils or macrophages might be less dependent on Notch activation. In those cells, intracellular pathways such as NF-κB might function to promote expression of PLA2G2A by neutrophils or macrophages might be less dependent on Notch activation.

Our microarray analysis also revealed a number of genes other than PLA2G2A that are regulated by NICD1 in IECs. Although the results did not show an upregulation of other genes specific to Paneth cells such as lysozyme or α-defensins, our quantitative RT-PCR confirmed that genes such as clustatin or spermidine/spermine N1-acetyltransferase were also upregulated upon Notch1 activation in LS174T cells (data not shown). Trefoil factor-1 may also promote Notch-mediated tissue regeneration because it is known to be a key factor in restitution (11). The group of genes shown in the present analysis was quite distinct from the previous microarray analysis comparing GSI-treated and untreated intestinal tissues (14, 27). Because we used an in vitro IEC-based assay, the group of genes identified can be recognized as candidates of the IEC-specific target genes of Notch. However, because only a limited number of genes were analyzed (up to 10,000 annotated genes) in the present study, a further analysis including a larger group of genes may elucidate additional genes that are regulated downstream of Notch.

In conclusion, Notch signaling acts as an indispensable intracellular signaling pathway in IECs, especially during tissue regeneration. It regulates not only the differentiation, but also the proliferation of IECs, and it also regulates the immune function of IECs. We have shown for the first time that such functions of Notch are also present in the human intestine, both under normal conditions and when tissue damage has occurred. The present study provides a novel molecular basis for the advanced understanding of the regeneration process in the human intestinal epithelia, which may be utilized to establish alternative therapies for refractory ulcers caused by various intestinal diseases.

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GRANTS


REFERENCES


