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Delta-like 1 expression promotes goblet cell differentiation in Notch-inactivated human colonic epithelial cells

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ABSTRACT

Notch signaling has previously been implicated in the regulation of the cell fate of intestinal epithelial cells. However, the expression and function of Notch ligands in the human intestine remain largely unknown. In the present study, we showed that Notch ligands Delta-like 1 (Dll1) and Delta-like 4 (Dll4) are expressed in a goblet cell-specific manner in human colonic tissue. Additionally, we found that Dll1 and Dll4 expression was regulated in-parallel with Atoh1 and MUC2, which are both under the control of the Notch-Hes1 signaling pathway. Because knockdown of Dll1 expression completely abrogated the acquisition of the goblet cell phenotype in Notch-inactivated colonic epithelial cells, we postulate that Dll1 might function as a cis-acting regulatory element that induces undifferentiated cells to become goblet cells. Our results suggest a link between Dll1 expression and human goblet cell differentiation that might be mediated by a function that is distinct from its role as a Notch receptor ligand.

Introduction

The mammalian intestinal epithelium consists of cells of four different lineages, each of which plays a distinct but indispensable role in supporting the function of intestinal tissues [1]. Goblet cells are one of three secretory-type cells that are present in the intestinal epithelium. They are characterized by the expression of MUC2 and play a crucial role in mucosal defense. Loss of goblet cell function leads to development of spontaneous colitis [2] and can subsequently lead to development of colon cancer [3]. Thus, maintenance of a proper number of functional goblet cells is required for homeostasis of the intestinal mucosal environment.

Recent studies in mice have revealed several key molecules that are required for the differentiation and maturation of intestinal goblet cells [4]. In one of these studies, Kim et al. found that Atoh1, which is expressed in goblet cells of the human colon, to be able to directly activate transcription of the human MUC2 gene [5]. In addition, our recent study showed that Atoh1 is a key regulator of goblet cell differentiation in the human intestine [6,7]. However, little is known about the upstream signals that regulate the Atoh1-MUC2 axis in goblet cells. We have previously shown that Wnt signaling plays a crucial role in the post-transcriptional regulation of Atoh1 [6,7]. Others have suggested that Notch-Hes1 signaling is involved in regulating the differentiation of intestinal cells into absorptive and goblet cells through a mechanism called lateral inhibition. [8]. In this model, absorptive cells arise from cells in which a high level of Notch activation is maintained. These cells express high levels of the Notch target gene Hes1. Conversely, secretory-type cells, including goblet cells, arise from cells expressing Notch ligands in which Notch signaling is inactivated. These cells instead express high levels of Atoh1, and inhibit neighboring cells from achieving the same cell fate.

Although this lateral inhibition model has been well-accepted as the mechanism that determines the cell fate of intestinal epithelial cells, little has been shown about where this process takes place in vivo. Our previous study, in which we used human tissues, showed that Notch is activated in crypt cells but is completely absent in goblet cells [9]. As described in the lateral inhibition model, the distribution of Notch-activated cells and goblet cells appeared side-by-side, suggesting that goblet cells might express Notch ligands and thereby activate the Notch receptors of neighboring cells. However, the expression and function of Notch ligands in the human intestine has never been described.

In the mammalian Notch system, there are five ligands that can activate the Notch receptor: Delta-like (Dll) 1, 3, and 4 and Jagged-1, and 2 [10]. These five ligands not only act in trans as ligands for the Notch receptor but might also act in cis to modulate

Abbreviations: NICD, Notch intracellular domain; Dll, Delta-like.

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intracellular Notch activity or gene expression [10]. Previous reports have shown that Notch ligands are expressed in the murine intestine [11]. In the mouse intestine, Dll1-expressing cells have been postulated to be progenitor cells of secretory-type epithelial cells, including goblet cells [12]. However, the functional role that Dll1 expression has on secretory and goblet cell differentiation remains to be elucidated.

In the present study, we sought to examine the expression and function of Notch ligands in human colonic tissue. We found that Dll1 and Dll4 were expressed in a goblet cell-specific manner and that Dll1 expression was necessary for acquisition of the goblet cell phenotype in Notch-inactivated colonic epithelial cells. Our results not only describe the important role that Dll1 expression plays in goblet cell differentiation, but they also suggest the possible existence of a cis-acting function of the Dll1 protein.

Materials and methods

Cell culture. Cell cultures and plasmid DNA transfections were performed as described previously [13]. The inhibition of endogenous Notch signaling was performed by addition of LY-411,575 (1 μM), which was synthesized according to the method of Wu et al. [14]. The cell lines expressing the Notch1 intracellular domain (TET-On NICD1 cells) and the cell line expressing FLAG-tagged Hes1 (TET-On FLAG-Hes1 cells) under the control of tetracycline or doxycycline (DOX, 100 ng/ml, Clontech) were generated from LS174T cells as described elsewhere [9]. Generated cells were supplemented with Blastcidin (7.5 μg/ml, Invitrogen) and Zeocin (750 μg/ml, Invitrogen).

Reverse transcription (RT)-PCR assays. Total RNA preparation and RT-PCR was performed as described previously [9]. We used primer sequences for human β-actin, G3PDH, MUC2, Hes1, and Atoh1 that have been previously described [6,9]. Primer sequences for the other genes and the number of PCR cycles that were used for semi-quantitative analysis are summarized in Supplemental Table 1. Results are presented as the means of the data collected from two rounds of assays. Each assay was performed in triplicate. We used paired two-sample Student's *t*-tests to analyse these data.

Plasmids. The expression plasmids used for this study were generated as described previously [9].

Immunoblot analysis. Immunoblot assays were performed as described previously [9]. We used anti-cleaved Notch1 (1:1000, Cell Signaling Technology), anti-Hes1 (1:4000, a kind gift from Dr. T. Sudo, Toray Industry), anti-Delta (recognizing both Dll1 and Dll4, 1:200, C-20, Santa Cruz Biotechnology), anti-Jagged-1 (1:200, H-114, Santa Cruz Biotechnology), and anti-β-actin (1:5000, SIGMA) primary antibodies.

Human intestinal tissue specimens. Human tissue specimens were obtained from patients who underwent endoscopic examination or surgery 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 both Yokohama Municipal General Hospital and Tokyo Medical and Dental University.

Immunohistochemistry. Immunohistochemistry using intestinal tissues was performed as previously described [15]. We used anti-Delta (recognizing both Dll1 and Dll4, 1:200, C-20, Santa Cruz Biotechnology) and anti-Jagged-1 (1:500, H-114, Santa Cruz Biotechnology) primary antibodies.

si-RNA mediated gene-knockdown. si-RNA-mediated gene knockdown was performed as previously described [13]. Briefly, a siRNA targeted to Dll1 mRNA (100 nM, Dharmacon) or a non-targeting siRNA (100 nM, Dharmacon) was transfected into cells using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol.

Results

Notch ligands are expressed in human colonic epithelial cells

Although several reports have shown that Notch ligands are expressed in the intestinal epithelium of rats and mice [11,16], they have not been shown to be expressed in human intestinal epithelial cells. Our previous report clearly showed that Notch1 activation is present in the crypt cells of the human intestinal epithelium [9]. These results suggested that Notch ligands might also be present in the human intestinal epithelium. Therefore, we first examined whether the RNA transcripts of Notch ligands could be detected within human intestinal biopsy specimens by semi-quantitative RT-PCR (Fig. 1A). Our results showed that other than Dll3, all Notch ligands are clearly expressed in the human colonic tissue. Analysis of the human colonic epithelial cell lines, LS174T and HT29, showed the same expression profile. These results suggested that of the five mammalian Notch ligands, four (Dll1, Dll4, Jagged-1, and Jagged-2) are expressed in human colonic tissue, possibly by epithelial cells.

To determine the precise distribution of Notch ligand-expressing cells, we performed immunohistochemical analysis using human colonic tissues. Immunostaining for Jagged-1 revealed that it was ubiquitously expressed in colonic crypt cells (Fig. 1B, right). In sharp contrast, immunostaining using an antibody which detects both Dll1 and Dll4 (anti-Delta) revealed selective staining positivity for goblet cells (Fig. 1B, left). Immunostaining for Jagged-2 was not successful. These results revealed that Jagged-1 and Dlls (Dll1 and Dll4) are both expressed by human colonic epithelial cells. However, Dll ligands are expressed by goblet cells and Jagged-1 is expressed by crypt cells. Therefore, these results suggest that the expression of Dll1 and Dll4 might have some functional significance in relation to the development of goblet cells within the human colonic epithelium.

Both the goblet cell phenotype and Dll ligand expression are downregulated upon activation of the Notch-Hes1 pathway in human colonic epithelial cells

Because our results suggested that Dll1 and Dll4 might play some role in the development of human goblet cells, we next examined the relationship between goblet cell differentiation and Dll ligands expression using a cell-based model.

In our previous report, we showed that forced expression of Notch1 intracellular domain (NICD1) suppressed the goblet cell phenotype of LS174T cells [9]. Using a sub-line of LS174T cells in

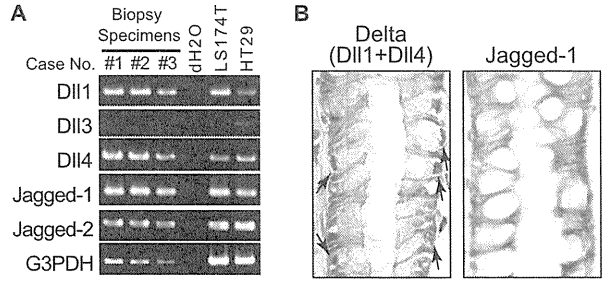

Fig. 1. Notch ligands are expressed in human colonic epithelial cells. (A) Expression of Notch ligands in human colonic biopsy specimens and in the human colonic epithelial cell lines LS174T and HT29 was analysed by semi-quantitative RT-PCR. (B) Expression of the Delta-like ligands Dll1, Dll4, and Jagged-1 in human colonic tissue was examined by immunohistochemistry. For the staining of Delta-like ligands, an anti-Delta antibody, which recognizes the c-terminal region of both Dll1 and Dll4, was used (Original magnification: 800×). The black arrows indicate goblet-shaped epithelial cells that stained positive for Dlls.

which we could induce NICD1 expression in a tetracycline- or doxycycline (DOX)-dependent manner (TET-on NICD1 cells), we examined whether the expression of Dll1 and Dll4 was regulated by intracellular Notch activity. Immunoblot analysis of TET-on NICD1 cells confirmed that expression of NICD1 was induced upon the addition of DOX (Fig. 2A). We also found that the mRNA expression of Hes1 was significantly upregulated upon the addition of DOX to TET-on NICD1 cells (Fig. 2B). Further analysis of gene expression showed that Dll1, Dll4, MUC2, and Atoh1 expression were all significantly downregulated in these cells. In sharp

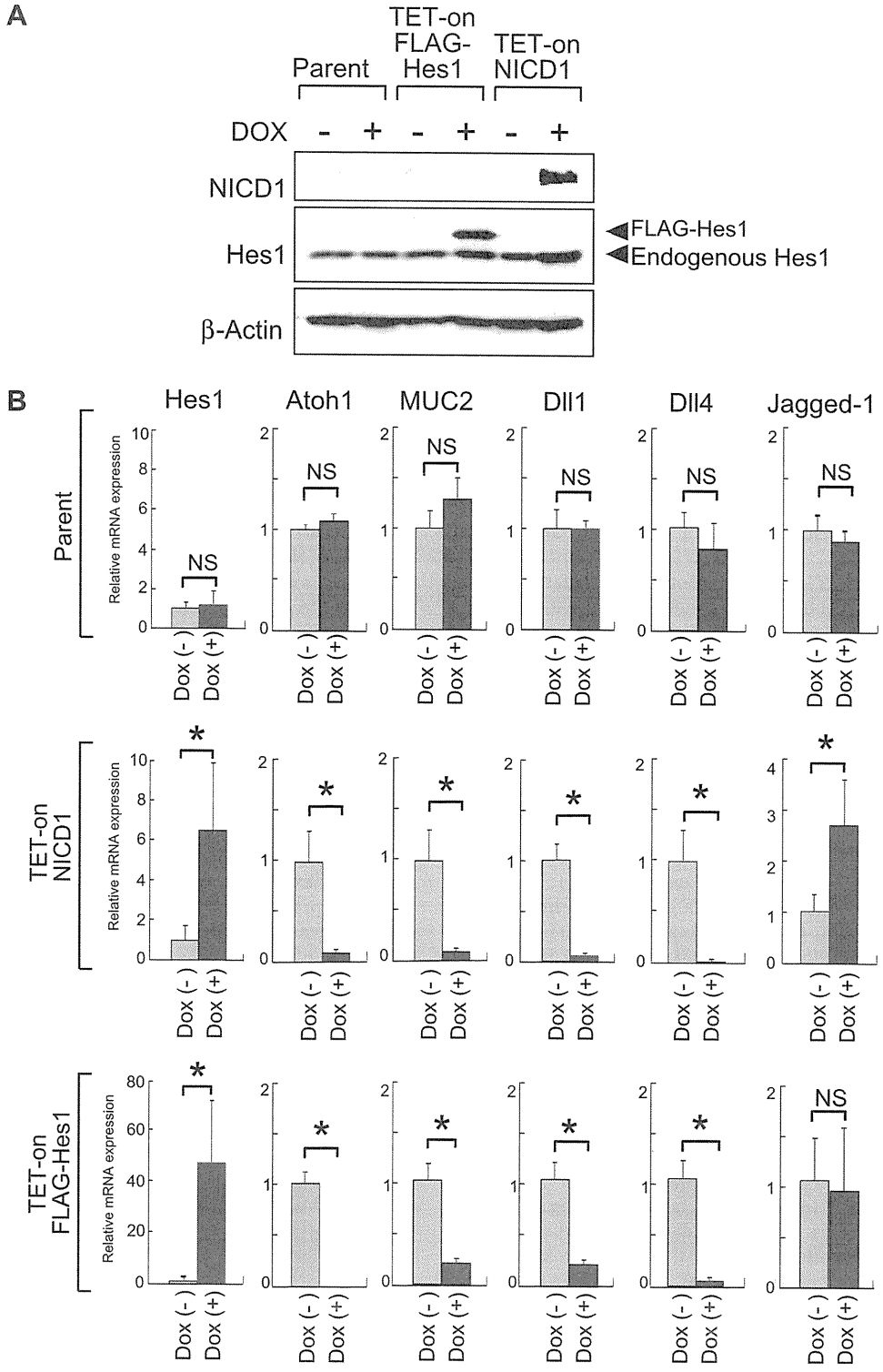

Fig. 2. Both the goblet cell phenotype and expression of Delta-like ligands are downregulated upon activation of the Notch-Hes1 pathway in human colonic epithelial cells. (A) Immunoblot analysis showing forced expression of Notch1 intracellular domain (NICD1) or FLAG-tagged Hes1 upon doxycycline (DOX) addition in TET-on NICD1 cells and TET-on Hes1 cells, respectively. Parent LS174T cells (Parent) served as the control. Cell lysates were prepared 72 h after DOX addition. (B) Expression of the indicated genes was analysed by quantitative RT-PCR. Data are means ± SD, normalized to the expression level of β-actin. $^{*}P < 0.05$. NS indicates that the comparison was not significant.

contrast, Jagged-1 demonstrated slight upregulation (up to 2.6-fold) when NICD1 expression was induced. These results suggested that expression of Dll1 and Dll4, but not Jagged-1 was regulated by intracellular Notch activity and that this occurred in-parallel with the expression of genes required for goblet cell differentiation.

To further confirm the role of the canonical Notch-Hes1 pathway, we generated another sub-line of LS174T cells in which we could induce FLAG-tagged Hes1 expression upon DOX addition (TET-on FLAG-Hes1 cells). Immunoblot (Fig. 2A) and quantitative RT-PCR analysis (Fig. 2B, which shows the total amount of both endogenous Hes1 mRNA and FLAG-Hes1 mRNA in these cells) confirmed that Hes1 expression is upregulated upon DOX addition to TET-on FLAG-Hes1 cells. We also found that the expression of Dll1 and Dll4, and the expression of Atoh1 and MUC2, was significantly downregulated upon the addition of DOX to TET-on FLAG-Hes1 cells (Fig. 2B). In sharp contrast, Jagged-1 expression remained unchanged. These results further confirmed that the in-parallel downregulation of Dlls and the genes required for goblet cell differentiation were mediated by the Notch-Hes1 axis in human colonic epithelial cells.

Both the goblet cell phenotype and Dll ligand expression are upregulated upon inactivation of the Notch-Hes1 signaling pathway in human colonic epithelial cells

The results of our previous experiments suggested that both Dll ligand expression and the acquisition of goblet cell phenotype are under control of the intracellular Notch-Hes1 signaling pathway. To further confirm that this regulatory system exists in human colonic epithelial cells, we next sought to examine the expression of Dll1 and Dll4 upon the induction of goblet cell differentiation. In our previous report, we showed that treatment with a gamma-secretase inhibitor, LY-411,575, completely blocked intracellular Notch activity and thereby induced significant upregulation of MUC2 expression in LS174T and HT29 cells [9]. Therefore, we examined the expression of Dll1 and Dll4 in this model of goblet cell differentiation.

Following the protocol used in our previous study, we analysed LS174T and HT29 cells by quantitative RT-PCR after they were treated with either DMSO or LY-411,575 (Fig. 3A). Treatment with LY-411,575 led to a significant downregulation of Hes1 mRNA

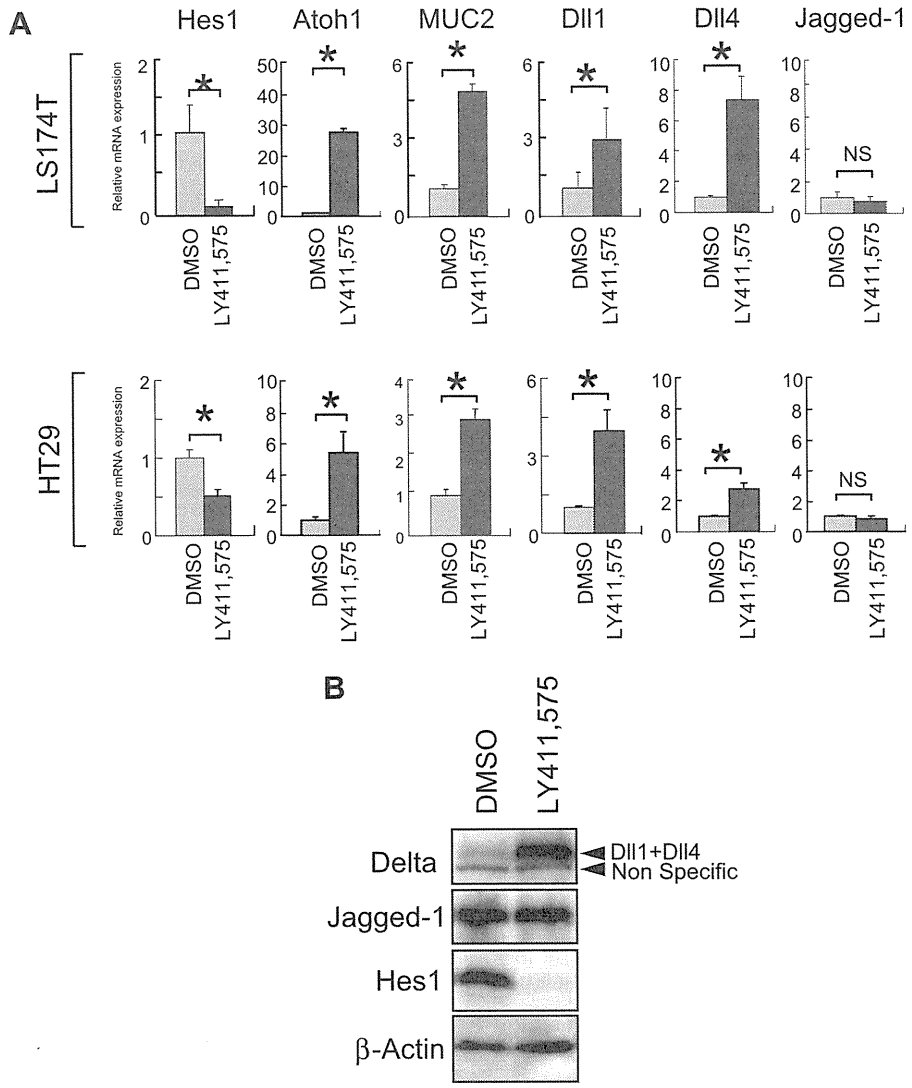

Fig. 3. Both the goblet cell phenotype and expression of Delta-like ligands are upregulated upon inactivation of the Notch pathway in human colonic epithelial cells. (A) Expression of the indicated genes in LS174T cells and in HT29 cells was analysed by quantitative RT-PCR. Total RNA was prepared after 72 h of culture with either LY-411,575 (1 μM) or DMSO. Data are means ± SD, normalized to the expression level of β-actin. *$P < 0.05$. NS indicates that the comparison was not significant. (B) Immunoblot analysis showing upregulation of Delta-like ligand proteins (total Dll1 and Dll4 content) upon Notch inactivation in LS174T cells. Cell lysates were prepared after 72 h of culture with either LY-411,575 (1 μM) or DMSO.

expression in both LS174T cells and HT29 cells, confirming that the intracellular Notch-Hes1 pathway had been inactivated. Consistent with our previous results, treatment with LY-411,575 upregulated Atoh1 and MUC2 expression in both cell lines. Under these conditions, expression of Dll1 and Dll4 was significantly upregulated in both cell lines, whereas Jagged-1 expression remained unchanged. This upregulation of Dlls was also confirmed at the protein level in LS174T cells, as immunoblot analysis of cells treated with LY-411,575 showed a significant increase in Dll protein levels (Fig. 3B, total Dll1 and Dll4 protein content). Thus, these results further confirmed that both expression of Dlls and the acquisition of the goblet cell phenotype are under the control of the intracellular Notch-Hes1 signaling pathway in human colonic epithelial cells. Also, these results are consistent with the results of our immunohistochemical analyses (Fig. 1B) in which we found that Dll1 and Dll4 were expressed in a goblet cell-specific manner, whereas Jagged-1 was expressed in a lineage-non-specific manner.

Upregulation of Dll1 is required for the upregulation of the goblet cell phenotype upon inactivation of the Notch-Hes1 signaling pathway in human colonic epithelial cells

The results from the experiments described above demonstrated that the expression of Dlls, Atoh1, and MUC2 are upregulated in-parallel upon inactivation of intracellular Notch signaling in human colonic epithelial cells. MUC2 has been reported to be one of the direct targets of Atoh1 [5]. Thus, it remains unknown whether the upregulation of Dll1 and Dll4 expression has any role in promoting cells to differentiate into goblet cells. If Dlls do play a role in this process, it also remains unknown whether they do so through the Atoh1-MUC2 axis.

To further study this process, we performed a knockdown experiment targeting Dlls to examine the functional role that Dll expression played in goblet cell differentiation. Despite our finding that treatment with gene-specific siRNAs significantly downregulated Dll1 and Dll4 expression at the mRNA level, our primary experiment showed a marked decrease of the total Dll1 and Dll4 protein content by the siRNA targeted for Dll1, but not for Dll4 (Supplemental Fig. 1). Thus, the dominant Dll protein expressed by LS174T cells appeared to be Dll1, not Dll4. Therefore, further analysis was performed using the Dll1-specific siRNA.

Combined treatment with LY-411,575 and a Dll1-targeted siRNA was performed using LS174T cells. Immunoblot analysis showed knockdown of Dll proteins in cells treated with Dll1-targeted siRNA (Fig. 4A). Also, Hes1 protein was completely undetectable in LY-411,575 treated cells. Quantitative RT-PCR analysis revealed that Dll1 mRNA expression was also clearly knocked down by treatment with the Dll1-specific siRNA, completely abrogating any increase in Dll1 expression upon treatment with LY-411,575 (Fig. 4B). In contrast, Dll4 expression was not affected by treatment with the siRNA targeted to Dll1. Knockdown of Dll1 significantly downregulated the expression of both Atoh1 and MUC2 in cells treated with DMSO alone (52% and 42% reduction for Atoh1 and MUC2, respectively). However, upon knockdown of Dll1 expression, a significant downregulation of Atoh1 and MUC2 expression was also observed in cells treated with LY-411,575 (64% and 70% reduction for Atoh1 and MUC2, respectively). Knockdown of Dll1 completely abrogated the upregulation of MUC2 expression by LY-411,575, suggesting that induction of the goblet cell phenotype in Notch-inactivated colonic epithelial cells is dependent upon the upregulation of Dll1 expression.

Based on these collective findings, we suggest a modified model describing the cell fate decision pathway that determines if a human intestinal epithelial cell will develop into an absorptive or goblet cell within the human intestinal epithelium (Fig. 4C). In this proposed model, the development of the absorptive cell phenotype

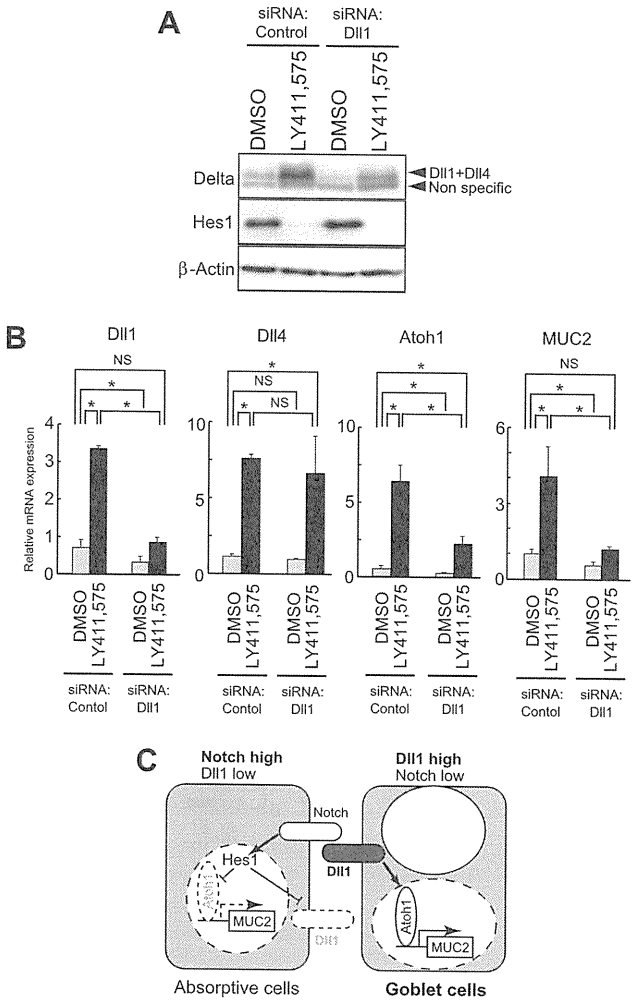

Fig. 4. Upregulation of Delta-like 1 is required for the upregulation of the goblet cell phenotype upon inactivation of the Notch pathway in human colonic epithelial cells. (A) siRNA-mediated knockdown of Delta-like 1 (Dll1) downregulated Delta-like ligand protein expression in LS174T cells. Cells were transfected with either Dll1-specific siRNA or non-targeting siRNA (Control) and cultured for 72 h with either LY-411,575 (1 μM) or DMSO. Immunoblot analysis was performed as described in Fig. 3B. (B) Expression of the indicated genes in LS174T cells was analysed by quantitative RT-PCR. Data are means ± SD, normalized to the expression level of β-actin. $^*P < 0.05$. NS indicates that the comparison was not significant. (C) A modified scheme of absorptive versus goblet cell differentiation that was derived from the present data.

is facilitated by the activation of the Notch-Hes1 pathway, the downregulation of both Atoh1 and Dll1 expression, and the subsequent downregulation of MUC2. However, the development of the goblet cell phenotype is facilitated by both Notch inactivation and Dll1 expression, as both are required for sufficient levels of Atoh1 and MUC2 expression. The present study raises the possibility that Dll1 might not only function in trans as a ligand that activates the Notch-Hes1 pathway in neighboring cells but that it might also function in cis to promote the differentiation of goblet cells in Notch-inactivated colonic epithelial cells.

Discussion

In the present study, we demonstrated for the first time that Dll1 and Dll4 are selectively expressed in goblet cells, whereas Jagged-1 is expressed in a lineage-non-specific manner within the human colonic epithelium. In a previous study, we showed that NICD1 is expressed in cells other than goblet cells, and it is

completely absent in mature goblet cells in the human colon [9]. Thus, the complementary expression of NICD1 and Dlls suggests that Dlls and not Jagged-1, take part in the lateral inhibition of Notch signaling within the colonic epithelium.

Previous reports by Crosnier et al. have shown that the genetic disruption of DeltaD (a zebrafish homologue of Dll) increases the number of secretory-type cells, including goblet cells, in the zebrafish intestine [12]. In their report, however, the increase in the number of secretory-type cells was minimal when the DeltaD protein was completely absent. In contrast, disruption of its ligand function alone resulted in upregulation of the DeltaD protein and a marked increase in the number of secretory-type cells in the intestinal epithelium. These findings are consistent with our present results showing that Dll1 might play a role in promoting goblet cell differentiation in human colonic epithelial cells that is distinct from its function as a ligand for Notch. Previous reports have consistently shown that Notch ligands may have functions other than acting merely as a ligand for Notch. Notch ligands are also cleaved by gamma-secretase upon binding to the Notch receptor, following which, the released intracellular domain localizes to the nucleus [10]. This intracellular domain of Notch ligands might function in cis as a transcriptional co-activator [17] and promote the expression of genes such as Atoh1 and MUC2. Consistently, our immunostaining of Dll1 and Dll4 revealed dominant staining in the nuclei of goblet cells (Fig. 1B). However, because our in vitro studies of goblet cell differentiation were based on the inactivation of Notch by a gamma-secretase inhibitor, it is possible that the observed function of Dll1 might not be dependent upon release of the intracellular domain. Notch ligands bound to the cell membrane may bind with transcriptional co-activators containing a PDZ-domain and could potentially regulate their function by keeping them away from the nucleus [10]. Thus, Dll1 might function as a regulator of intracellular transcription through such a mechanism.

Another finding of the present study was that the expression of the Dlls, Atoh1, and MUC2 were under the control of the Notch-Hes1 axis. A previous report found that Dll1 was a direct target of Hes1 in mice [18]. Our results showed that Dll1 and Dll4 might be one of the downstream targets of Hes1 in human colonic epithelial cells. Such regulation of Dlls, Atoh1 and MUC2 by the Notch-Hes1 axis emphasizes their importance in lateral inhibition, thereby contributing to promote absorptive cell differentiation (Fig. 4C).

Although we found that Dll1 is required for proper goblet cell development in Notch-inactivated cells, Dll4 appeared to be of less importance in this context. Knockdown of Dll1 downregulated total Dll1 and Dll4 protein content in LS174T cells (Fig. 4A). Thus, Dll4 might be expressed in low levels as compared to Dll1 in colonic epithelial cells. Also, previous in vivo studies have shown that the administration of a neutralizing antibody for Dll4 had no effect on murine intestinal epithelium [19]. Thus, although the importance of Dll4 expression remains to be elucidated, as compared to Dll1, its role may be minimal with regard to the induction of goblet cell differentiation.

In conclusion, Dll1 expression is upregulated upon Notch inactivation, which is required for the proper acquisition of the goblet cell phenotype. Further studies focusing on a potential cis-acting function of the Dll1 protein might reveal a yet unknown link between Dll1 expression and the regulation of goblet cell differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.048.

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Increased expression of lipocalin-type-prostaglandin D synthase in ulcerative colitis and exacerbating role in murine colitis

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Increased expression of lipocalin-type-prostaglandin D synthase in ulcerative colitis and exacerbating role in murine colitis

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Hokari R, Kurihara C, Nagata N, Aritake K, Okada Y, Watanabe C, Komoto S, Nakamura M, Kawaguchi A, Nagao S, Urade Y, Miura S. Increased expression of lipocalin-type-prostaglandin D synthase in ulcerative colitis and exacerbating role in murine colitis. *Am J Physiol Gastrointest Liver Physiol* 300: G401–G408, 2011. First published December 16, 2010; doi:10.1152/ajpgi.00351.2010.—The pathogenesis of ulcerative colitis (UC) is unclear, but enhancement of disease activity by usage of nonsteroidal anti-inflammatory drugs suggests involvement of prostanoid in its pathophysiology. However, biological effect of prostaglandin (PG) D_2 on intestinal inflammation remains unknown. We investigated the expression of enzymes for PGD_2 synthesis, prostaglandin D synthase (PGDS), and its relation to the activity of colitis in UC patients. The role of lipocalin-type PGDS (L-PGDS) using a murine colitis model was also assessed. Tissue samples were obtained by colonic biopsies from patients with UC. Expression levels of mRNAs for L-PGDS and hematopoietic-type PGDS were investigated by quantitative RT-PCR. COX-2 and L-PGDS expression was investigated by immunohistochemistry. Localization of L-PGDS expression was also determined by in situ hybridization. In experimental study, mice were treated with dextran sodium sulfate in the drinking water to induce colitis. The degree of colonic inflammation was compared with L-PGDS$^{-/-}$ mice and control mice. The level of L-PGDS mRNA expression was increased in UC patients in parallel with disease activity. Colocalization of L-PGDS and cyclooxygenase (COX) 2 was observed in lamina proprial infiltrating cells and muscularis mucosa in UC patients. The level of hematopoietic PGDS mRNA expression did not differ from control mucosa. Dextran sodium sulfate treatment to L-PGDS$^{-/-}$ mice showed lower disease activity than control mice. We reported for the first time the presence of L-PGDS in the COX-2-expressing cells in the mucosa of active UC patients and that only L-PGDS increased with disease activity. An animal model study suggests that PGD_2 derived from L-PGDS-expressing cells plays proinflammatory roles in colitis.

prostaglandin D_2; dextran sodium sulfate; chemoattractant receptor-homologous molecule expressed on Th2 cells; ulcerative colitis

THE PATHOGENESIS OF ULCERATIVE colitis (UC) is unclear, but enhancement of disease activity by usage of cyclooxygenase (COX) inhibitor suggests involvement of prostanoid in its pathophysiology (10, 11). In an animal model of colitis, administration of nonsteroidal anti-inflammatory dugs to IL-10$^{-/-}$ mice enhanced disease activity (21). Modulation of disease activity by a difference in dietary fat intake also suggests involvement of prostanoid in its pathophysiology, since polyunsaturated fatty acid is a substrate for prostanoid synthesis (22, 28).

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Major prostanoid, both prostaglandin E_2 (PGE_2) and Prostaglandin D_2 (PGD_2) are synthesized from prostaglandin H_2 (PGH_2) by specific enzyme, prostaglandin E synthase (PGES) or prostaglandin D synthase (PGDS) respectively. PGD_2 is synthesized by hematopoietic PGD synthase (H-PGDS) or lipocalin-type PGDS (L-PGDS) (5, 30, 31, 32). PGH_2, a precursor of both PGD_2 and PGE_2, is synthesized by prostaglandin H synthase (PGHS), also known as COX. Which prostanoid, PGE_2 or PGD_2, the COX-positive cells synthesize is determined by expression of PGES or PGDS in their cells (14, 17). The balance between PGDS and PGES is a major determinant of immune response in atherosclerotic plaque (6). Divergent roles of PGE_2 or PGD_2 on the immune system are reported. PGD_2 is a well known immunomodulator in peripheral tissues (13, 16, 18), invoking airway inflammation (18, 27), inhibiting platelet aggregation (33), and causing peripheral vasodilatation (2, 29).

In the intestinal immune physiological system, roles of PGE_2 are well studied. People suggest that most biological effects by usage of COX inhibitor are the result of a decrease in PGE_2 (4). Increased disease activity to dextran sodium sulfate (DSS)-induced colitis on EP4$^{-/-}$ mice suggests a protective role of PGE_2 in this mice model (15). Direct administration of PGE_2 to the colonic lumen of DSS-induced colitis ameliorates colitis (25). However, in contrast to accumulating reports of the biological effect of PGE_2, that of PGD_2 in intestinal disease is limited. There has been no report about PGD_2 in UC. We previously reported that chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) positive cells infiltrated to colonic mucosa of UC patients (19). Recently, it is reported that CRTH2 serves as a receptor for PGD_2, and it induces chemotaxis in Th2 cells, eosinophils, basophils, and macrophages in response to PGD_2. Thus, we hypothesized that PGD_2 possibly involves changes of colonic inflammation in UC.

In this study, we investigated the expression of enzymes for PGD_2 synthesis and the relationship between levels of their expression and disease activity in UC patients. Furthermore, to elucidate the role of L-PGDS in colonic inflammation, disease activity of DSS-induced colitis was compared in L-PGDS knockout mice with that in wild-type mice.

MATERIAL AND METHOD

Patients. Tissue samples were obtained by colonic biopsies from 24 patients with UC (13 men, 11 women; mean age 36.2 yr; range 21–59 yr). UC was diagnosed on the basis of standard diagnostic criteria. Ten patients had proctosigmoiditis, six had left-sided colitis, and eight had total colitis. Medications being taken at the time of colonoscopy were as follows: 5–20 mg/day prednisolone in 9 patients; 1.5–4.5

g/day sulfasalazine or 1.5–2.25 g/day 5-aminosalicylic acid in 22 patients; and 15–30 mg/day 6-mercaptpurine in 2 patients. Twelve patients received a combination of these at the time of colonoscopy. Biopsy specimens were collected from the inflamed mucosa (inflamed) during endoscopy. In addition, a biopsy specimen was collected from the noninflamed mucosa (noninflamed) from nine patients with UC (5 men and 4 women; mean age 36.2 yr; range 21–54 yr).

The histological scores of hematoxylin and eosin (H&E) staining sections were assessed using the criteria previously reported by Matts (1 = normal appearance; 2 = some infiltration of the mucosa or lamina propria with either round cells or polymorphs; 3 = much cellular infiltration of the mucosa, lamina propria, and submucosa; 4 = presence of crypt abscesses, with much infiltration of all layers of the mucosa; 5 = ulceration, erosion, or necrosis of the mucosa, with cellular infiltration of some or all of its layers) (20).

As controls, colonic biopsy specimens were obtained from 16 patients with colonic polyps (7 men and 9 women; mean age 44.4 yr; range 22–57 yr). Biopsy specimens of their colonic mucosa were histologically normal.

Informed consent was obtained from all patients. The protocols of the research were approved by the Ethical Committee of the National Defense Medical College and conformed to the provisions of the World Medical Associations Declaration of Helsinki as revised in 1975.

Immunohistochemical examination. Immunohistochemical examination was performed using the labeled streptavidin biotin method. Colonic tissues were fixed in periodate, lysine-paraformaldehyde solution and were vertically embedded in optimum cutting temperature compound (Miles, Elkhart, IN) before being frozen in dry ice and acetone. Well-oriented 6-μm-thick cryostat sections were transferred to amino-propyltri-ethoxycylan-coated slides and air dried for 1 h at 20°C. They were then washed in PBS (pH 7.4) containing 1% Triton X-100 for 5 min, and sections were incubated in 10% normal goat serum in PBS. Monoclonal antibodies used in this study were as follows: antihuman COX-1 antibody (CX111, Cayman Chemical no. 160110, Ann Arbor, MI), antihuman COX-2 antibody (Rabbit polyclonal IgG, Cayman Chemical no. 160107), and antihuman L-PGDS antibody (Mab-1B7). Mab-1B7 was mouse monoclonal antibody raised against recombinant human L-PGDS as described previously (8). Isotype- and species-matched IgG was used for control. After treatment with each antibody, they were visualized by treatment with avidin-biotinilated peroxidase complex for 1 h at 37°C, and then sections were immersed in 3,3′-diaminobendine tetrahydrochloride solution containing 0.03% H_2O_2. Sections were observed under a microscope (BX60; Olympus, Tokyo, Japan).

In situ hybridization. In situ hybridization was performed by using a RiboMap Kit and the Discovery automatic staining module (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions. Digoxigenin (DIG)-labeled RNA probe for human L-PGDS was prepared as follows. The coding region of human L-PGDS was subcloned into pCR-Script Amp SK(+) vector (Stratagene). The inserted fragment was amplified with T7 and SP6 primers, and purified by a Microspin column (GE Healthcare) or agarose gel electrophoresis. DIG-labeled cRNA probes were synthesized using T7 (antisense) or SP6 (sense) RNA polymerase according to the manufacturer's manual (Roche Diagnostics), analyzed on an agarose gel, and purified by a Chroma Spin Column (BD Biosciences, San Jose, CA). The labeling efficiency was determined by dot-blotting with a dilution series of the DIG-labeled cRNA probes and control RNA fragments of known DIG contents. The sections were hybridized with 10 ng of RNA probe in Ribohybe hybridization solution (Ventana Medical Systems) containing 500 ng of tRNA at 70°C for 3 h and incubated with horseradish peroxidase-conjugated anti-DIG antibody (DakoCytomation) for 30 min, followed by dinitrophenol (DNP)-conjugated tyramide. Tyramide was then activated by adding H_2O_2 for 20 min. After being heated at 85°C for 10 min and washed, the sections were incubated with anti-DNP antibody for 30 min, avidin for 1 h, and biotin for 1 h (Endogenous Biotin Blocking Kit; Ventana Medical Systems) to block the nonspecific staining due to endogenous biotin retrieved by the heating procedure. The sections were then incubated with biotin-conjugated anti-rabbit antibody for 10 min and streptavidin-conjugated alkaline phosphatase for 16 min (AmpMap with TSA; Ventana Medical Systems). The chromogen reaction of alkaline phosphatase was performed with a BlueMap Kit (Ventana Medical Systems). The sections were counterstained with nuclear fast red (ISH Red Counterstain; Ventana Medical Systems) and observed under an ECLIPSE E600 microscope (Nikon).

Double immunolabeling. For double staining with COX-2 (Cayman Chemical no. 160107) and mouse monoclonal anti-L-PGDS antibody (Mab-1B7), after the primary antibodies had been applied, the sections were sequentially incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) and biotinylated anti-mouse IgG antibody (5 μg/ml; Jackson ImmunoResearch) followed by Alexa Fluor 594-conjugated streptavidin (5 μg/ml; Invitrogen). The sections were observed under an Axiovert 100M microscope connected to a Zeiss laser-scanning microscope 510META (Carl Zeiss, Jena, Germany).

Quantitative RT-PCR. Total RNA was extracted from biopsy specimens using the RNeasy Mini isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was quantified and checked for purity by spectrophotometry at 260 and 280 nm. Aliquots of total RNA were reversely transcribed using SuperScriptII Reverse Transcriptase (Invitrogen) and subsequently amplified by PCR using the Taq DNA polymerase (Promega, Madison, WI). TaqMan RT-PCR was performed in duplicate for each sample using the ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster City, CA). Amplifications were generated by 10 min at 95°C and then 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 62°C for 1 min by using the TaqMan universal PCR Master Mix kit and Assays-on-Demand Gene Expression probes [L-PGDS, Hs00183950; H-PGDS, Hs00168748; murine PGES (mPGES)-1, Hs00610420; Applied Biosystems]. To standardize the quantitation of selected genes, β-glucronidase (GUS) from each sample was quantified on the same plate with the target genes by using the TaqMan β-GUS Control Reagents kit (Applied Biosystems). We also used the TaqMan human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Control Reagents kit (Applied Biosystems) for endogenous control. We confirmed that those endogenous genes expressed equally in our samples. For quantitative RT-PCR of murine intestinal mucosa, the same procedure was performed using Mm0133061 for murine L-PGDS and Mm0452015 for murine mPGES-1. To standardize the quantitation, the TaqMan GAPDH Control Reagents kit was used.

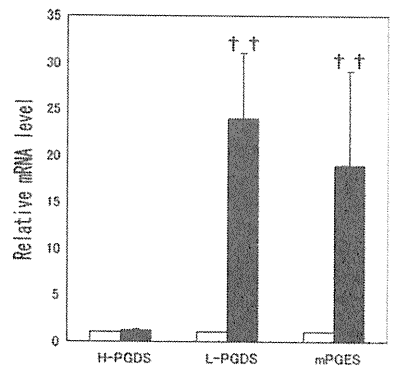

Fig. 1. Lipocalin (L)-type prostaglandin D synthase (PGDS), hematopoietic PGD synthase (H-PGDS), and murine prostaglandin E synthase (mPGES) mRNA expressions in the colonic mucosa of the control patients and patients with ulcerative colitis as determined by quantitative RT-PCR. Significant increases in L-PGDS and mPGES were observed in colonic mucosa of ulcerative colitis patients. □, Control mucosa; ■, mucosa of ulcerative colitis patients. ‡$P < 0.05$ vs. control.

Induction of colitis. Mice deficient in L-PGDS from a background of C57/Bl6 (L-PGDS-deficient mice) were generated at Osaka Bioscience Institute (Osaka, Japan), as reported in detail by Eguchi et al. (9) Male L-PGDS knockout (KO) mice and appropriate wild-type controls (18–20 g, 8 wk old) were housed in wire-mesh-bottom cages in specific pathogen-free condition in our animal laboratory center. The care and use of laboratory animals were in accordance with the National Institutes of Health guidelines. The experimental protocol was approved by the Animal Research Committee of the National Defense Medical College. DSS (mol wt 40,000) was purchased from ICN Biochemicals (Cleveland, OH). DSS was dissolved in water and adjusted to a concentration of 3.5% (wt/vol). For induction of colitis, mice received DSS in drinking water for 7 days, followed by a 7-day interval with normal drinking water. Mice used for experiments were age-matched and had received DSS treatment simultaneously.

Assessment of colitis. Body weight was determined every two or three days. At the end of the study, the colon was removed and opened longitudinally. The length of colon was measured as a parameter for colonic inflammation. The colon was fixed in 10% buffered formalin. Tissues were embedded in paraffin, and they were stained with H&E. The histological damage score was measured by the method of cryptscoring by Cooper et al. (7): *grade 0*, intact crypt; *grade 1*, loss of the basal one-third of the crypt; *grade 2*, loss of the basal two-thirds of the crypt; *grade 3*, loss of entire crypt with the surface epithelium remaining intact; *grade 4*, loss of both the entire crypt and surface epithelium.

Colonic myeloperoxidase activity assay. The tissue was homogenized in 50 mM phosphate buffer containing 0.5% hexadecyl-trimethyl-ammonium bromide, pH 6.0 (Sigma-Aldrich, St. Louis, MO) on ice using a Polytron homogenizer and centrifuged at 2,000 rpm for 10 min at 4°C. Myeloperoxidase (MPO) was measured by the Quanti-Chrom Peroxidase Assay Kit (BioAssay Systems) in the colorimetric assay procedure using the supernatant. One unit of MPO activity was defined as enzyme necessary to degrade 1 μmol of H_2O_2 to H_2O per minute at 25°C. Results are expressed as international units per gram tissue protein.

Statistical analysis. All results are expressed as means ± SD. Differences between two groups were evaluated using the Mann-Whitney *U*-test. Between more than two groups, differences were evaluated using one-way ANOVA and a post hoc test. Probabilities <0.05 were considered to be significant.

RESULTS

mRNA expression of L-PGDS, H-PGDS, and mPGES-1 in colonic mucosa. To determine the role of PGD_2 in ulcerative colitis (UC), we first investigated whether PGDS, a key molecule for PGD_2 synthesis, is expressed in inflamed colonic mucosa or not. In control patients, we could detect the expression of both types of PGDS (L-PGDS and H-PGDS) in the colonic mucosa as determined by the quantitative RT-PCR

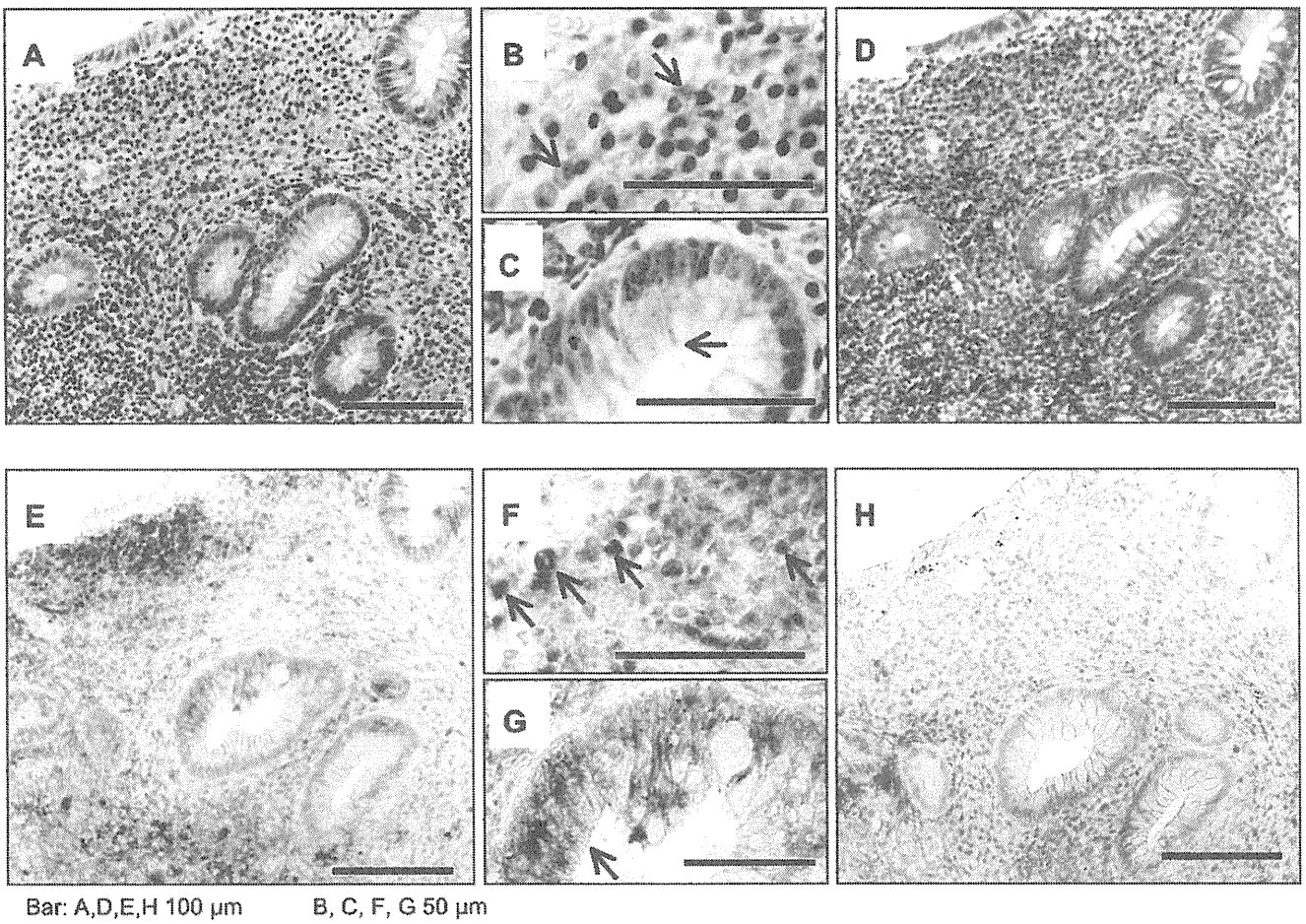

Fig. 2. Immunohistochemical staining (*A*) and in situ hybridization (*E*) of L-PGDS in the colonic mucosa from an ulcerative colitis patient. L-PGDS immunoreactivity was observed in the infiltrating inflammatory cells and epithelial cells (arrows in *B* and *C*). No positive staining of the colonic mucosa was detected by applying a rabbit IgG (*D*). L-PGDS antisense RNA probe signal was observed in the same area (arrows in *F* and *G*). When sense RNA probe was applied, no signal was detected in colonic mucosa (*H*). Scale bar = 100 μm for *A*, *D*, *E*, and *H* and 50 μm for *B*, *C*, *F*, and *G*.

method. In UC patients, the expression of L-PGDS mRNA showed a 20-fold increase compared with that in the control mucosa (Fig. 1). On the other hand, H-PGDS mRNA expression was not increased in UC patients, suggesting that PGD_2 derived from L-PGDS plays a greater role than does H-PGDS in the pathophysiology of UC. Thus, we examined mRNA expression of L-PGDS in the following studies. As it was reported in the previous study, the expression of mPGES-1 was also significantly increased in the colonic mucosa of UC patients compared with that in the control mucosa (Fig. 1).

Histological localization of L-PGDS and COX-2 in colonic mucosa. We next investigated the immunohistochemical distribution of L-PGDS and COX-1/2 in the colonic mucosa (Fig. 2, *A–D*, and Fig. 3). In control patients, only marginal L-PGDS immunoreactivity was detected in the colonic mucosa (data not shown). In the colonic mucosa of UC patients, L-PGDS immunoreactivity was clearly observed in inflammatory cells in the lamina propria (Fig. 2, *A* and *B*), in the epithelium (Fig. 2, *A* and *C*), and in the lamina muscularis mucosa (Fig. 3*A*). Consistent with the immunohistochemical observation, in situ hybridization of L-PGDS anti-sense probe showed that epithelial cells (Fig. 2, *E* and *G*) and inflammatory cells in the lamina propria showed positive reactivity (Fig. 2, *E* and *F*). COX-2 immunoreactivity was observed in the lamina muscularis mucosa (Fig. 3*B*) and infiltrating inflammatory cells (Fig. 3*E*) in UC patients. Comparison of the serial sections of the same tissues showed the colocalization of L-PGDS and COX-2 in the muscularis musosa (Fig. 3, *A* and *B*) and lamina proprial inflammatory cells (Fig. 3, *D* and *E*), suggesting that these cells are producing PGD_2. On the other hand, COX-2 expression in the epithelial cells was marginal (Fig. 3*E*), suggesting that epithelial cells cannot produce a significant amount of PGD_2. COX-1 expression was not observed in the epithelium or in the lamina proprial area of colonic mucosa in UC patients (Fig. 3, *C* and *F*).

L-PGDS expression in a different degree of inflammation. To clarify that L-PGDS expression is relevant to pathophysiology of UC, we investigated the relationship between its expression and disease activity. The levels of expression of L-PGDS were compared between the patients of Matts 2 and the patients >Matts 3. As shown in Fig. 4, the levels of expression of L-PGDS mRNA significantly increased in severely inflamed mucosa (Matts >3) than that of mildly inflamed mucosa (Matts 2).

To clarify whether there is a significant difference in the L-PGDS expression between actively inflamed mucosa and the quiescent mucosa, we compared them in the same patient. As shown in Fig. 5, the levels of expression of L-PGDS mRNA were significantly greater in the inflamed mucosa than in the quiescent mucosa ($P < 0.01$).

Disease activity of L-PGDS-deficient mice after DSS treatment. DSS treatment to mice induced colitis in wild-type mice. The majority of wild-type mice with colitis induced by DSS feeding had loose stools (87.5%) and bloody stools (37.5%). The body weight of wild-type mice after 7 days of DSS treatment was significantly decreased compared with untreated controls. Body weight decreased even further during the normal drinking water period following DSS treatment (Table 1 and Fig. 6*A*). We killed mice at *day 14* when mice showed a marked decrease in body weight. Macroscopically, in wild-type mice, DSS treatment resulted in marked intestinal damage, shortening of colonic

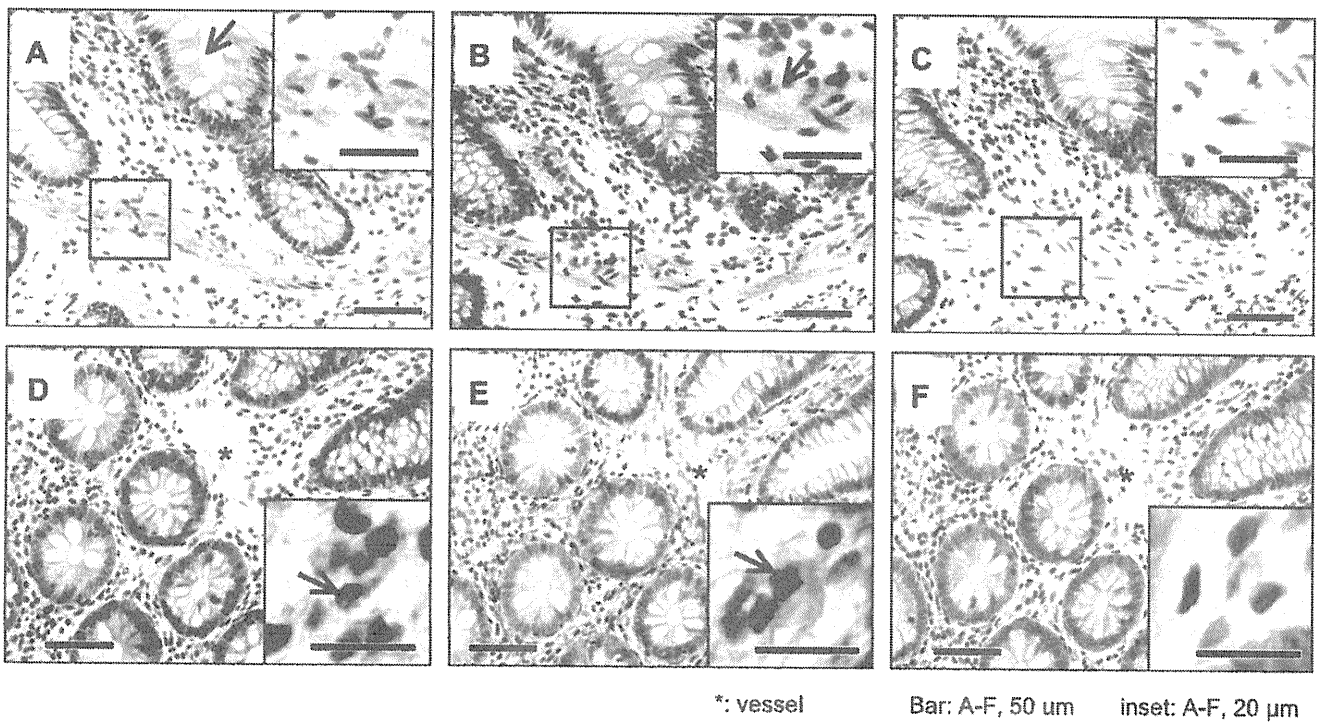

Fig. 3. Immunohistochemical staining of L-PGDS, cyclooxygenase (COX)-1, and COX-2 in the colonic mucosa. In the colonic mucosa from ulcerative colitis patients, strong L-PGDS immunoreactivity was observed in the lamina muscularis mucosa as well as epithelial cells (arrows in A) and infiltrating inflammatory cells in the lamina propria (arrows in *D*). COX-2 immunoreactivity in ulcerative colitis patients was observed in the lamina muscular mucosa (arrows in *B*) and infiltrating inflammatory cells (arrows in *E*) in the same way as L-PGDS. COX-1 immunoreactivity was not observed (*C* and *F*). Scale bar = 50 μm. Inset: scale bar = 50 μm.

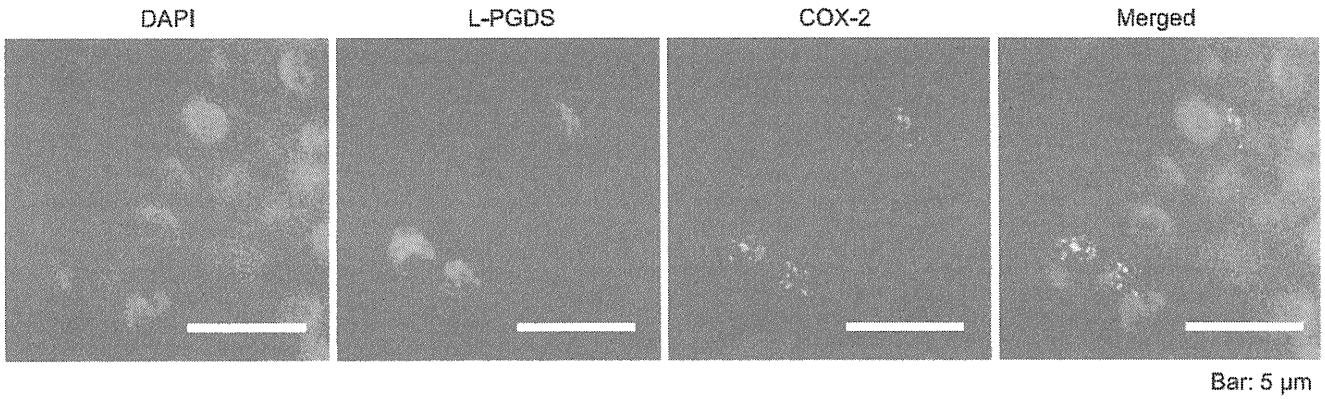

Fig. 4. Expression of L-PGDS and COX-2 in the lamina proprial infiltrating cells of colonic mucosa of UC patient observed by double immunolabeling and laser scanning confocal microscopy. A confocal microscopy demonstrates colocalization of L-PGDS (red) and COX-2 (green) in the same cells. Scale bar = 5 μm.

length, increased thickness of colonic walls with mucosal edema, and loss of normal longitudinal folds in the lower half of the colon. Histologically, the lesions induced by DSS were characterized by colitis with many infiltrating cells consisting of mononuclear cells in both lamina propria and submucosa. We observed the formation of lymphoid follicles, an increase in matrix with fibroblasts, and crypt distortion in DSS-induced colonic mucosa. Loss of entire crypts and erosions were observed in some portions (Fig. 6*B*). In contrast, DSS treatment to L-PGDS$^{-/-}$ mice resulted in lower disease activity than that of control mice. The minority of L-PGDS-deficient mice with DSS treatment had loose stools and bloody stools. Loss of body weight and histological damage scores were also attenuated in L-PGDS$^{-/-}$ mice (Table 1). L-PGDS$^{-/-}$ mice without DSS treatment did not show any clinical and histological difference from untreated wild-type mice.

Figure 6*A* shows the time course of body weight change caused by DSS treatment in wild-type mice and L-PGDS$^{-/-}$ mice. The decrease in body weight of L-PGDS$^{-/-}$ mice was significantly ameliorated compared with the wild-type mice after 14 days of treatment. Increased thickness of colonic mucosa with inflammatory cell infiltration was significantly ameliorated in L-PGDS$^{-/-}$ mice (Fig. 6*C*).

L-PGDS and PGES expression in murine colonic mucosa. Figure 7 shows mRNA expressions of L-PGDS and PGES in murine colonic mucosa of wild-type and L-PGDS-deficient mice and the effect of DSS treatment. The basal level of mPGES-1 mRNA in colonic mucosa was not different between control mice and L-PGDS$^{-/-}$ mice. A significant increase in levels of PGES expression was observed in the inflamed colonic mucosa in DSS-induced wild-type mice compared with that in control mice . The level of L-PGDS mRNA expression of colonic tissue was measured. The degree of L-PGDS mRNA expression was low in the colonic mucosa of control mice; however, a remarkable increase in expression levels of L-PGDS was induced in DSS-treated wild-type animals in the same way as was seen in human UC. Lack of expression of L-PGDS mRNA in L-PGDS-deficient mice was confirmed. In addition, in L-PGDS-deficient mice, levels of mPGES-1 mRNA expression in colonic mucosa showed no significant difference between DSS-treated control mice and DSS-treated L-PGDS$^{-/-}$ mice.

MPO activity in colonic mucosa. We measured colonic MPO activity to clarify whether L-PGDS KO mice may have a defect in neutrophil infiltration that may underlie the protection from DSS-induced damage in these mice. MPO values were not different between wild-type mice and L-PGDS$^{-/-}$ mice in the control condition (7.7 ± 2.4 and 8.2 ± 3.5 IU/μg protein, respectively). DSS treatment significantly increased MPO activity both in wild-type mice and L-PGDS$^{-/-}$ mice, but those were not different (28.4 ± 9.2 and 29.6 ± 8.5/μg protein, respectively).

DISCUSSION

Previously, we reported that CRTH2-positive cells infiltrate to inflamed colonic mucosa in UC patients. In this study, we for the first time reported that colonic mucosa from UC patients expressed L-PGDS but not H-PGDS in the COX-2-positive inflammatory cells in lamina propria. In addition, the level of

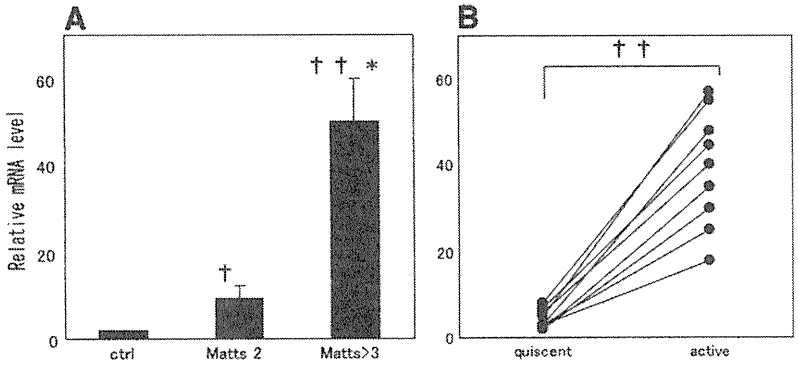

Fig. 5. Comparison of L-PGDS mRNA expression in the colonic mucosa from ulcerative colitis patients between the weakly inflamed mucosa group (Matts 2) and the highly inflamed group (Matts 3 and more). †$P < 0.05$ vs. control. ††$P < 0.01$ vs. control. *$P < 0.05$ vs. Matts 2 group. *A*: difference in L-PGDS mRNA expression in the colonic mucosa from ulcerative colitis patients between two different sites, the quiescent colonic mucosa and inflamed colonic mucosa in the same patients. *B*: paired samples from 9 ulcerative colitis patients were assayed for L-PGDS mRNA expression. ††$P < 0.01$ vs. quiescent mucosa.

Table 1. *Clinical features and histological damage score in murine colitis*

	Wild Type Untreated	L-PGDS$^{-/-}$ Untreated	Wild Type + DSS	L-PGDS$^{-/-}$ + DSS
Loose stool	0/8	0/8	7/8	4/12
Bloody stool	0/8	0/8	3/8	2/12
Body wt, g	28.1 ± 2.2	28.3 ± 2.8	17.8 ± 2.8†	20.7 ± 1.5‡
Damage score	0	0	2.58 ± 0.44†	1.55 ± 0.38‡

Data for loose stool and bloody stool are the no. of animals/total animals. Values for body weight and damage score are averages ± SD. *Evaluation was made after dextran sodium sulfate (DSS) treatment. L-PGDS, lipocalin-type prostaglandin D synthase. †$P < 0.05$ vs. wild type untreated. ‡$P < 0.05$ vs. wild type + DSS.

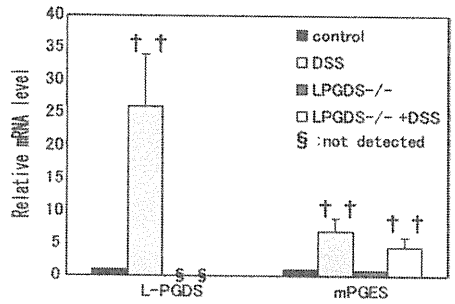

Fig. 7. L-PGDS and mPGES-1 mRNA expression in the colonic mucosa of control mice and L-PGDS$^{-/-}$ mice with and without DSS treatment. ††$P <$ 0.01 vs. control group.

expression of L-PGDS increased in relation to disease activity and was higher in actively inflamed mucosa than quiescent mucosa in the same patients. In inflamed colonic mucosa of the murine colitis model, an increased level of L-PGDS mRNA expression was observed as well as human UC. Finally, the activity of colonic inflammation induced by DSS treatment was decreased in L-PGDS-deficient mice. Collectively, we propose that PGD2 derived from L-PGDS is involved in pathophysiology of UC, and its role is suggested to be proinflammatory by the animal model study.

Generation of PGD_2 in immunologically activated intestinal mucosa of rats is already reported by another group (12). However, in contrast to our present findings, Ajuebor et al. (1) reported a protective role of exogenous administration of PGD_2 in the trinitrobenzene sulfonic acid (TNBS)-induced colitis model, suggesting that an excessive dose of PGD_2 plays a protective role in colitis (1). The exact reasons for the discrepancy between their study and ours are not known; however, it may be explained by two possibilities. One is due to the difference in the model of colitis between the two studies. In their model, the level of PGDS mRNA expression was decreased rapidly after induction of colitis, and the concentration of PGD_2 only increased in a short period until 3 h. The concentration of PGD_2 did not show correlation with MPO activity, and PGD2 or PGDS already decreased at the time colitis was induced. This may be due to an acute direct effect of intracolonically administered TNBS on the intestinal mucosa. On the other hand, we confirmed an increased level of L-PGDS mRNA expression in murine inflamed colonic mucosa as was seen in UC patients, suggesting that our colitis model is more suitable to explore the role of PGD_2 in colitis. The second possibility is due to the fact that they administered PGD_2 exogenously. In vivo, PGD_2 is synthesized from PGH_2 by PGDS only within COX-positive cells because the life period of PGH_2 outside of cells is very short. It is considered that spatial distance between PGD_2-receptor positive cells and PGD_2-producing cells is important physiologically. Thus, the effect of exogenously administered PGD_2 might exert a nonspecific effect and could not reflect the actual role of PGD_2 in inflamed colon. These opposite effects of prostanoid in pathophysiology of colitis are seen in the case of PGE_2. In this study, the level of mPGES expression significantly increased in colonic mucosa of UC patients, suggesting that PGE_2 is involved in pathophysiology of UC. The protective role of PGE_2 in colitis is suggested by many papers. For instance, it was reported that disease activity was enhanced by DSS treatment in prostaglandin E receptor-deficient mice (15). On the contrary, an exogenously administered high dose of PGE_2 analogs induced a harmful effect in the TNBS colitis model with an increase in IL-23 and IL-17 (26). It is also explained that exogenously administered PGE_2 played some nonphysiological roles to prostaglandin E receptor-positive cells that were distant from PGE_2-producing cells.

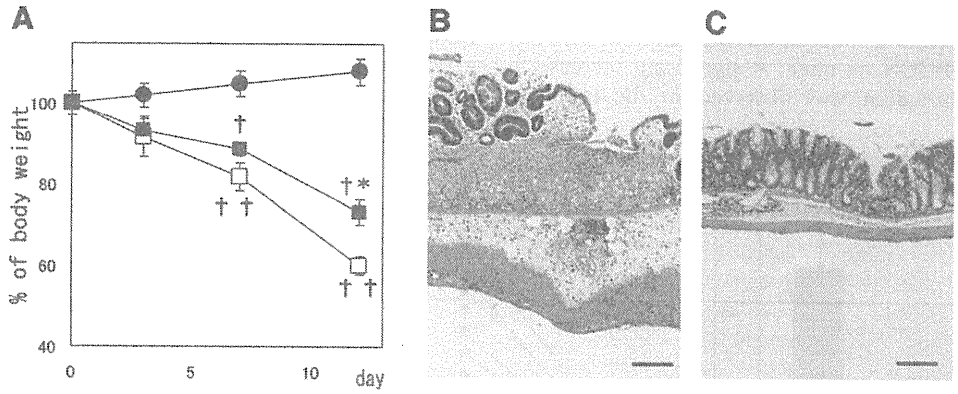

Fig. 6. Time course of body weight change caused by dextran sodium sulfate (DSS)-treated mice between wild-type mice and L-PGDS$^{-/-}$ mice (*A*). ●, Control mice; ■, DSS treatment to L-PGDS$^{-/-}$ mice; □, DSS treatment to wild-type mice. The body weight of mice in the DSS to wild-type mice group was significantly decreased compared with the control group. The level of decrease in body weight by DSS treatment in L-PGDS$^{-/-}$ mice was ameliorated compared with the wild-type mice group. Data are expressed as means ± SD. ††$P < 0.01$ vs. control. †$P < 0.05$ vs. control. *$P < 0.05$ vs. wild-type mice with DSS treatment. Representative hematoxylin and eosin staining of murine colitis in wild-type mice (*B*) and in L-PGDS$^{-/-}$ mice (*C*). Scale bar = 100 μm.

Our results suggest the proinflammatory role of L-PGDS in the development of colitis in clinical and experimental study. It may be due to PGD_2-dependent action or PGD_2-independent action. In this study, lamina proprial infiltrating cells and lamina muscularis mucosa expressed both COX-2 and L-PGDS in UC patients, suggesting that these cells produce PGD_2. As a PGD_2-dependent action, it plays chemoattractant roles for inflammatory cells such as Th2 cells and eosinophils through CRTH2 receptor to the colonic mucosa.

In addition to the chemoattractant effect, PGD_2 plays an immunomodulatory effect on inflammatory cells through the prostaglandin D receptor. In addition, PGD_2 is converted to 15dPGJ2 and plays immunomodulatory roles through PPAR-gamma thereafter. However, recent studies revealed that both PGD_2 and 15d-PGJ(2) appear to possess not only anti-inflammatory activities but also a proinflammatory potential depending on its concentration and the activation state of the target cell. For instance, PGD_2 or 15dPGJ2 stimulated the proliferation in the leukocyte cell line THP-1 at lower concentrations, whereas they inhibited the proliferation through the induction of apoptosis at high concentrations (3). In addition to the immune system, $15dPGJ_2$ is suggested to play proinflammatory roles by inducing apoptosis of several human epithelial cell lines, including gastric, lung, colon, prostate, and breast. These proinflammatory potentials of PGD_2 are in accordance with our results of lower disease activity in L-PGDS-deficient mice after DSS treatment.

Previous study showed that COX-2 positive inflammatory cells such as macrophages have the capacity to express both PGDS and PGES and they change the expression of PGDS or PGES in a inflamed condition. It is reported that the balance between PGDS and PGES in macrophages is a major determinant of the immune response in atherosclerotic plaque (6). Thus we had expected that PGDS deficiency might increase the level of expression of PGES, leading to a decrease in the activity of colitis; however, the level of expression of mPGES-1 was not different between control mice and L-$PGDS^{-/-}$ mice in a control condition and even after induction of colitis. Thus, we conclude that indirect involvement of PGE_2 in the ameliorating effect against colitis in L-PGDS-deficient mice is less in this study.

Recently, PGD_2-independent roles of L-PGDS are reported. We cannot exclude the possibility that PGD_2-independent action of L-PGDS also might be involved in colitis because, in this study, epithelial cells of a UC patient only expressed L-PGDS without significant COX-2 expression, suggesting that those cells play some PGD_2-independent roles. Increased expression of L-PGDS inhibits cellular growth in smooth muscle cells (23). Elevated L-PGDS activity contributes to apoptosis in various cell types, including epithelial cells, neuronal cells, and vascular smooth muscle cells (24). Collectively, there is a possibility that increased expression of L-PGDS itself but not through PGD_2 in colonic epithelial cells also may be responsible for increased activity of colitis. More detailed studies are necessary to elucidate the mechanism regarding the involvement of L-PGDS in colitis; bone marrow chimeras would help to determine the difference of contribution of L-PGDS in epithelial cells and hematopoietic cells.

In conclusion, the results of the present study demonstrate the appearance and possible involvement of one important prostaglandin-converting enzyme, L-PGDS, in colitis. The attenuation of colitis in L-PGDS deficiency suggests the potential usefulness of selective L-PGDS inhibitor for inflammatory bowel disease treatment.

GRANTS

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DISCLOSURES

There is no conflict of interest.

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Physiological stress exacerbates murine colitis by enhancing proinflammatory cytokine expression that is dependent on IL-18

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Physiological stress exacerbates murine colitis by enhancing proinflammatory cytokine expression that is dependent on IL-18

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Matsunaga H, Hokari R, Ueda T, Kurihara C, Hozumi H, Higashiyama M, Okada Y, Watanabe C, Komoto S, Nakamura M, Kawaguchi A, Nagao S, Sekiyama A, Miura S. Physiological stress exacerbates murine colitis by enhancing proinflammatory cytokine expression that is dependent on IL-18. *Am J Physiol Gastrointest Liver Physiol* 301: G555–G564, 2011. First published June 30, 2011; doi:10.1152/ajpgi.00482.2010.—Psychological stress is an environmental factor considered to be a precipitating factor of inflammatory bowel disease. Interleukin (IL)-18 plays a role in stress-induced aggravation in some diseases. The aim of this study was to establish a model of murine colitis exacerbated by psychological stress and to clarify the role of IL-18 in this model. Male C57Bl/6 mice and IL-18$^{-/-}$ mice were used for this study. The mice received dextran sulfate sodium (DSS) for induction of colitis. Some mice were exposed to psychological stress using a communication box. Body weight, colonic length, and histological inflammation were measured for assessment of colitis. Tumor necrosis factor (TNF)-α and IL-18 expression in the colon and IL-18 expression in the adrenal gland were analyzed using real-time PCR. The effect of anti-IL-18 antibody was also investigated. Effects of TNF-α and IL-18 on cytokine expressions were studied using the colonic epithelial cell line LS174T. Induction of psychological stress in DSS-treated wild-type mice significantly exacerbated colitis with enhanced expression of proinflammatory cytokines and IL-18. However, induction of psychological stress in DSS-treated IL-18$^{-/-}$ mice did not aggravate colitis compared with that in the IL-18$^{-/-}$ group given only DSS treatment. Stress-induced aggravation of colitis was ameliorated significantly by anti-IL-18 antibody treatment. IL-18 did not enhance TNF-α-induced expression of intercellular adhesion molecule-1 or IL-8 in LS174T. We established a model of colitis exacerbated by psychological stress. Psychological stress enhanced IL-18 expression and plays a proinflammatory role in stress-induced aggravation of colitis.

psychological stress; colitis; interleukin-18; mouse

THE PATHOGENSIS OF INFLAMMATORY bowel disease (IBD) is still unknown, and it has a complex multifactorial etiology comprising genetic (32) and environmental factors (2, 47) that are associated with dysregulation of the mucosal immune system. Psychological stress is an environmental factor that has long been suggested to contribute to the pathophysiology of IBD. Indeed, during the 1950s, ulcerative colitis was regarded as a psychosomatic disease (10). While clinical observations have provided anecdotal evidence (36, 37), there have been few prospective studies in which the involvement of stress in exacerbation or precipitation of inflammatory relapse in IBD patients was examined. A prospective cohort study showed that long-term perceived stress increases the risk of exacerbation of ulcerative colitis (19). Another recent study has suggested that several stressors can increase the rate of relapse in patients with IBD (21). In animal studies, it has been shown that captivity stress and readjustment to a novel social environment cause spontaneous colitis in cotton-topped tamarins (*Sanguinus Oedipus*) (8, 11). Repeated exposure to various stressors over a relatively short period, including restraint (12, 26), and a combination of cold and restraint stresses (33) also exacerbate colonic inflammation in rats. In addition, reactivation of completely resolved acute colitis after a combination of restraint and sonic stress has been reported (35). In agreement with the above-reported results, stress has been shown to affect the pathogenesis of diseases in immunological animal models (6, 17, 27, 45). However, although the involvement of somatic stress in colitis has been studied, the involvement of psychological stress has not been investigated.

Psychological and/or physical stresses affect host defenses comprising neuronal, endocrine, and immune systems (9, 18). A variety of cytokines [e.g., IL-1β, IL-6, and tumor necrosis factor (TNF)-α] are upregulated by stresses (9, 23, 53), suggesting that the cytokines are involved in interference with host defenses (38, 49, 54). Moreover, it has been reported that stress can modulate intestinal inflammatory responses through multiple routes, including neural and neuroendocrine pathways, the hypothalamus-pituitary-adrenal (HPA) axis, and the release of cortical-releasing hormone (13). Communication between stress and the gut is via the HPA axis and sympathetic nervous system axis (13). Because stress has various effects on gastrointestinal functions (22, 43), including intestinal barrier function, luminal bacteria adherence, and mucosal immune functions, stress may directly and indirectly influence the balance of proinflammatory and anti-inflammatory cytokines in the intestine.

The pleiotropic interleukin (IL)-18 is thought to be one of the crucial mediators (15, 20, 42, 46, 50) since it activates various signal pathways, including those engaged in cell proliferation/survival (4, 28). Subsequent studies have demonstrated that IL-18 has multiple biological activities (7, 28, 29), including induction of Fas ligand, elevation of cytolitic activity of T cells (29), and production of Th2 cytokines (14, 52). IL-18 also activates Toll-like receptor 2 (3) and myeloid differentiation protein 88 (1). Thus, IL-18 is involved in the production of both Th1 and Th2 cytokines (7, 14, 28, 29). Conti et al. (5) showed that IL-18 mRNA is expressed in the adrenal gland in response to adrenocorticotropic hormone (ACTH) and cold stress. Sugama et al. (44) reported differential IL-18 promoter

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usage in the adrenal gland and immune cells with adrenal gland-specific expression of IL-18 mRNA induced by ACTH, suggesting that IL-18 may be induced in the adrenal gland during stress. These findings led us to speculate that IL-18 plays a pivotal role in the regulation of mucosal barrier functions under psychologically stressful conditions.

We hypothesized that psychological stress modulates colonic inflammation through IL-18. To determine the validity of our hypothesis, we investigated whether psychological stress exacerbates colonic inflammation and whether blockade of IL-18 modulates this inflammation.

MATERIALS AND METHODS

Animals. IL-18 gene knockout (IL-18$^{-/-}$) mice, originally from the Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME) and wild-type mice on a C57Bl/6 background (Japan Clea, Tokyo, Japan) were maintained on a diet of standard laboratory chow (Oriental Yeast Manufacturing, Tokyo, Japan) and in specific pathogen-free conditions. Both wild-type and IL-18$^{-/-}$ mice were 8–10 wk of age, sex-matched, and housed four to five per cage, allowed free access to food and tap water, and were maintained in an animal colony at the National Defense Medical College (NDMC), according to the policies and recommendation of the NDMC Animal Care and Use Committee. This study protocol was approved by the Animal Ethical Committee of NDMC (no. 05093).

Induction of colitis. Dextran sulfate sodium (DSS, mol wt 40,000) was purchased from ICN Biochemicals (Cleveland, OH). DSS was dissolved in water and adjusted to a concentration of 3% (wt/vol). For induction of colitis, mice received DSS treatment for 5 days followed by 5 days with normal drinking water (24, 25).

Exposure to psychological stress. Psychological stress was produced using a communication box (Muromachi Kikai, Tokyo, Japan) that permitted one set of animals to receive a physical stressor in the form of a series of brief (10 s) electric shocks delivered using an electronic shock generator (31) over a period of 2 h. To induce psychological stress, DSS-treated mice or controls given only water were placed in compartments adjacent to those receiving the electric shock, and psychological stress was induced by their ability to view and hear the responses of the animals that received the physical stress (electric shock). To minimize distress, physically stressed animals were given only one 2-h session of electric shock and were then killed. DSS-treated mice and water controls exposed to psychological stress were exposed to different groups of physically stressed mice each day for five consecutive days after treatment with DSS or water. These groups of mice exposed to psychological stress were not given food or water during the time when mice of the physically stressed group received shock exposure. We note that the 5 days of repeated psychological stress was the minimum required to induce stress-associated weight loss in DSS-treated mice, and this same exposure did not reduce weight in water-treated mice. Mice that were not exposed to psychological stress were also deprived of food and water for 2 h/per day for 5 days.

Assessment of colitis. Body weight was determined every 2 or 3 days. At the end of the study, the colon was removed and opened longitudinally. The length of colon was measured as a parameter for colonic inflammation. The colon was fixed in 10% buffered formalin. Tissues were embedded in paraffin, and they were stained with hematoxylin and eosin (H & E). Histological damage score was

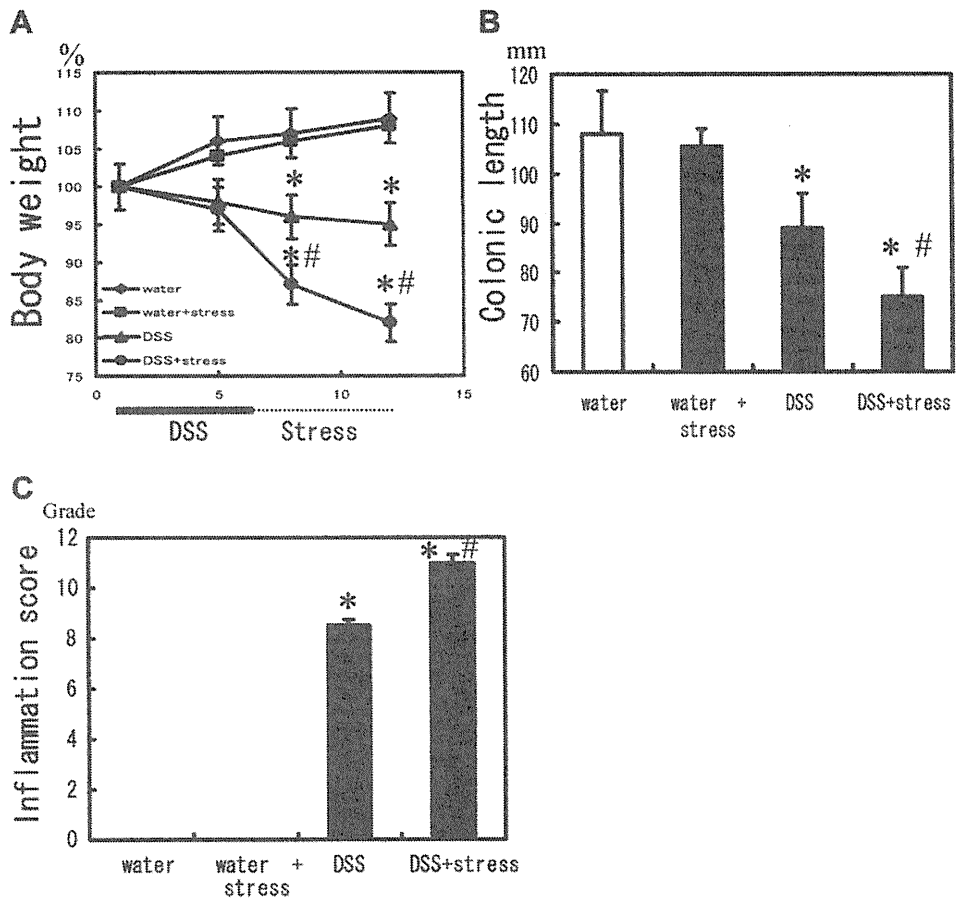

Fig. 1. Effects of dextran sodium sulfate (DSS) and psychological stress on percent change of body weight (*A*), colonic length (*B*), and grade of mucosal inflammation (*C*) in each group of wild-type mice. Water, a control group administered only water; Water+Stress, a group administered water and exposed to psychological stress; DSS, a group administered DSS; DSS+Stress, a group administered DSS and exposed to psychological stress. DSS treatment induced colitis, and exposure to psychological stress after DSS treatment aggravated colitis. Stress treatment alone did not induce colitis. Data are shown as means ± SE; $n = 6$ experiments. $P < 0.05$ vs. the water group (*) and vs. the DSS group (#).

measured by the method of crypt damage and inflammation scoring by Williams et al. (51), ranging from 0 to 14. Total colitis is the sum of the four subscores. Inflammation severity was scored as follows: 0, none; 1, mild; 2, moderate; and 3, severe. Inflammation extent was scored as follows: 0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transmural. Crypt damage was scored as follows: 0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, crypts lost; and 4, crypts and surface epithelium lost. Percent involvement was scored as follows: 0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 75–100%. In some groups, the number of infiltrating immune cells was calculated and quantified per millimeter of muscularis mucosa.

Treatment with anti-IL-18 neutralizing antibody. In some groups, mice were treated with antibody daily from *day 6* to *day 10* for 5 days 3 h before psychological stress administration. Aniti-IL-18 antibody (93–10C, rat IgG; MBL, Nagoya, Japan) or isotype- and species-matched Ig (rat IgG; Chemicon Intern, Temecula, CA) was administered intraperitoneally at the dose of 25 μg/mouse.

Real-time PCR. Intestinal mucosa was removed after mice were killed. Total mRNA was extracted by using the RNeasy Mini isolation kit (Qiagen). TaqMan reverse transcription PCR was performed in duplicate for each sample using the ABI PRISM 7000 Sequence Detector (Applied Biosystems). Primers and probes used in this study were purchased from Applied Biosystems: TNF-α (Mm00443258), IL-18 (Mm00434225), and IL-6 (Mm0044619).

IL-18 expression in the adrenal glands. Adrenal glands in the wild-type mice were removed after DSS treatment or exposure of psychological stress or both. IL-18 mRNA expression in the adrenal gland was analyzed using real-time PCR.

IL-18, IL-18 receptor, and proinflammatory cytokine expression in the colonic epithelial cell line. The human colon cancer cell line LS-174T was purchased from the American Type Culture Collection (ATCC CL188). Recombinant human IL-18 was from MBL, and recombinant human TNF-α was purchased from R&D (Minneapolis, MN). LS-174T was grown in MEM containing 10% FBS at 37°C,

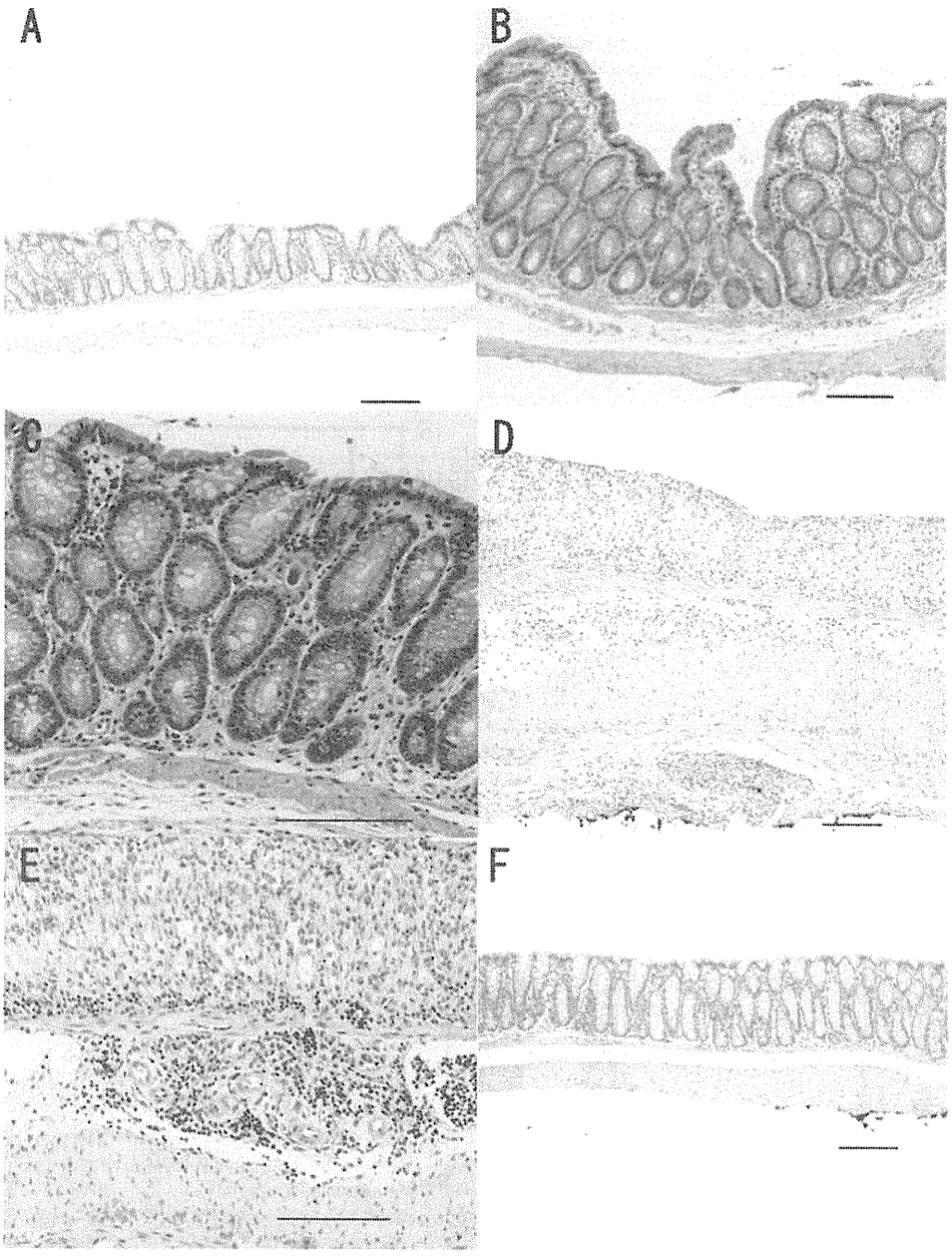

Fig. 2. Effects of DSS and psychological stress on representative hematoxylin and eosin (H & E) sections of the distal colon in each group of wild-type mice. *A*: a control group administered only water. *B* and *C*: a group administered DSS. *D* and *E*: a group administered DSS and exposed to psychological stress. *F*: a group administered water and exposed to psychological stress. DSS treatment induced mild colitis with immune cell infiltration. Exposure to psychological stress after DSS treatment aggravated colitis with severe immune cell infiltration, severe epithelial damage, thickening of the muscularis propria, and focal transmural inflammation. Stress treatment alone did not induce colitis. Bars indicate 100 μm. *A*, *B*, *D*, and *F*: ×10 objective. *C* and *E*: ×20 objective.

95% air, and 5% CO_2. Cells were serum-starved and then treated with TNF-α at the concentration of 20 ng/ml or IL-18 at the concentration of 20 ng/ml for 6 h. Total RNA was harvested by using the RNeasy Mini isolation kit (Qiagen). Real-time quantitative RT-PCR was carried out by using the following primer and probe set: IL-18 (Hs01038788; Applied Biosystems), IL-18 receptor-1 (Hs00977691; Applied Biosystems), IL-8 (Hs00174103; Applied Biosystems), and intercellular adhesion molecule-1 [ICAM-1 (Hs00174103; Applied Biosystems)].

Statistical analysis. Data are expressed as means ± SE. Differences between groups were examined for statistical significance using one-way factorial ANOVA and Fisher's protected least-significant difference test. *P* values of 0.05 or less were considered to be statistically significant. Statistical analyses were performed using the Statcel2 software (Addinsoft; OMS, Tokyo, Japan).

RESULTS

Exacerbation of DSS-induced colitis by psychological stress. Although psychological stress is thought to aggravate human colitis, there have been no studies to test whether psychological stress aggravates colitis in a murine model of colitis (20, 22). We investigated whether psychological stress aggravates DSS-induced colitis. Figure 1*A* shows the effects of DSS and psychological stress on percent change in body weight of wild-type mice. Mice in the water-administered group gained weight throughout the study period. Mice in the water-administered plus psychological stress-exposed group also gained weight throughout the study period. Mice in the DSS group and the DSS plus psychological stress group showed significant weight loss compared with weight in the water group. The degree of body weight loss in the DSS plus psychological stress group was significantly higher than that in the DSS group. Figure 1*B* shows the effects of DSS and psychological stress on colonic length of wild-type mice. Colonic length in the DSS group and that in the DSS plus psychological stress group were significantly decreased compared with that in the water group. The degree of decrease in the DSS plus psychological stress group was significantly higher than that in the DSS group. Stress treatment alone did not decrease colonic length. Figure 2 shows a representative H & E section of the distal colon and grade of colonic inflammation in each group of wild-type mice. There were very few inflammatory cells and intact crypts in the colonic mucosa of mice in the water group (Fig. 2*A*) and water plus stress group (Fig. 2*F*). On the other hand, mild colitis characterized by an increased number of inflammatory cells, decreased number of crypts, and increased submucosal thickness was observed in the DSS without stress group (Fig. 2, *B* and *C*). Severe colitis characterized by total loss of crypt formation and numerous inflammatory cells in the lamina propria was observed in the DSS plus psychological stress group (Fig. 2, *D* and *E*). Figure 1*C* shows the grade of colonic inflammation in each group of wild-type mice. Histological damage score in the DSS group and that in the DSS plus psychological stress group were significantly increased compared with that in the water group. Histological damage score in the DSS plus psychological stress group was significantly higher than that in the DSS without psychological stress group.

Next we studied whether expression of proinflammatory cytokines in the colonic mucosa was involved in psychological stress-induced modification of colitis activity. Figure 3 shows relative mRNA expression levels of TNF-α (*A*) and IL-18 (*B*) in the colonic mucosa of each group of wild-type mice. Psychological stress treatment alone did not increase TNF-α expression levels. TNF-α expression levels in the DSS group and the DSS plus psychological stress group were increased significantly compared with that in the water group. In addition, TNF-α expression level in the DSS with psychological stress group was significantly higher than that in the DSS without psychological stress group. These results suggest that *1*) TNF-α is involved in the pathogenesis of colitis, which is consistent with previous reports and *2*) TNF-α is involved in the psychological stress-induced aggravation of colitis. IL-18 was also expressed in the colonic mucosa in the control water group. The level of IL-18 expression in the water plus stress group was significantly higher (2.7-fold higher) than that in the control water group. DSS treatment alone tended to increase IL-18 expression, although the increase was not significant. IL-18 expression level in the DSS plus psychological stress group was significantly increased compared with the levels in the control water group and the DSS group. These results suggest that IL-18 is involved in the psychological stress-induced aggravation of colitis.

Increase of IL-18 expression in adrenal glands after exposure to psychological stress. It is generally accepted that psychological stress increases IL-18 expression in adrenal glands through the HPA axis. We investigated whether IL-18 expression increased in our psychological stress model by using a communication box. Figure 4 shows relative mRNA expression of IL-18 in the adrenal glands of each group of wild-type mice. IL-18 was expressed in the control group. IL-18 expression levels in the psychological stress group and

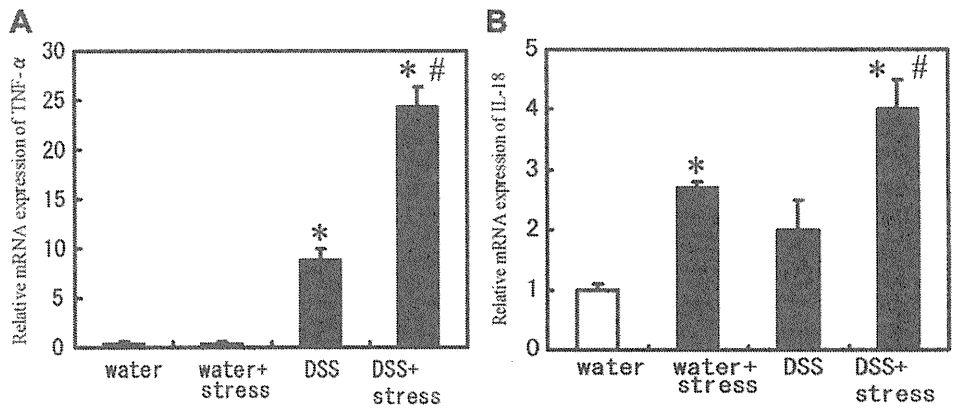

Fig. 3. mRNA expression of tumor necrosis factor (TNF)-α (*A*) and interleukin (IL)-18 (*B*) in the colonic mucosa of each group of wild-type mice. TNF-α expression levels in the DSS group and the DSS+Stress group were increased significantly compared with that in the water group. The TNF-α expression level in the DSS+Stress group was significantly higher than that in the DSS group. IL-18 expression level in the Water+Stress group was significantly higher than that in the water group. IL-18 expression level in the DSS+Stress group was significantly higher than the levels in the water group and the DSS group. Data are shown as means ± SE; $n = 6$. $P < 0.05$ vs. the water group (*) and vs. the DSS group (#).