Original Article

Reciprocal regulation of protein arginine deiminase 2 and 4 expression in the colonic mucosa of ulcerative colitis

Yasuo Otsuka,[1] Yasuhiro Masuta,[1] Kosuke Minaga,[1] Natsuki Okai,[1] Akane Hara,[1] Ryutaro Takada,[1] Sho Masaki,[1] Ken Kamata,[1] Hajime Honjo,[1] Kouhei Yamashita,[2] Masatoshi Kudo,[1] and Tomohiro Watanabe[1,*]

[1]Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan
[2]Department of Hematology and Oncology, Kyoto University Graduate School of Medicine, 54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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Neutrophils express protein arginine deiminase 2 and PAD4, both of which mediate the citrullination of target proteins to induce production of neutrophil extracellular traps. Although PAD-dependent NETs trigger inflammatory bowel disease, the mechanisms governing the expression of PAD2 and PAD4 are poorly understood. In this study, we tried to clarify expression mechanisms of PAD2 and PAD4 in the colonic mucosa of patients with ulcerative colitis and Crohn's disease. Administration of Cl-amidine, a pan PAD-inhibitor, attenuated the development of dextran sodium sulfate-induced colitis, the effects of which were accompanied by reduced IL-6 and TNF-α production by colonic lamina propria mononuclear cells upon exposure to Toll-like receptor ligands. The mRNA expression of colonic *PAD2* and *PAD4* was negatively and positively correlated with disease activity and pro-inflammatory cytokine responses in patients with UC, respectively. Reciprocal regulation of *PAD2* and *PAD4* mRNA expression was observed in the colonic mucosa of UC patients, but not in those of CD patients. *PAD4* mRNA expression was correlated with disease activity and pro-inflammatory cytokine responses in patients with CD. Collectively, these data suggest that reciprocal regulation of PAD2 and PAD4 expression is associated with disease activity in UC patients.

***Key Words*: Crohn's disease, ulcerative colitis, PAD2, PAD4**

Inflammatory bowel disease (IBD) is classified into Crohn's disease (CD) and ulcerative colitis (UC).[1,2] Production of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-12, IL-23, and tumor necrosis factor (TNF)-α, produced mainly by macrophages and dendritic cells, underlies the immunopathogenesis of CD and UC.[3] Immune cells accumulated in the gastrointestinal tract of patients with CD and UC are composed of T cells, B cells, plasmacytes, macrophages, dendritic cells, and neutrophils.[1,2] Predominant accumulation of neutrophils results in crypt abscesses, one of the characteristic pathological findings in UC.[2,4] Neutrophil infiltration correlates with disease activity in both CD and UC, whereas impaired neutrophil function is associated with CD.[4–7] Thus, activation of neutrophils is involved in the immunopathogenesis of both CD and UC.

Protein arginine deiminase (PAD)2 and PAD4 are expressed in neutrophils.[4,8] PAD2 and PAD4—two major PAD isozymes—promote autoimmunity in rheumatoid arthritis (RA) through the citrullination of target proteins.[8] Citrullination of histones by PAD4 in neutrophils is indispensable for production of neutrophil extracellular traps (NETs), which are composed of histones, decondensed DNA, and neutrophil granules.[9] PAD2 also participates in NET production in the absence of PAD4.[8–10] Although NETs contribute to host defense against microbial infection, recent studies provide evidence that excessive production of NETs triggers the development of autoimmunity.[9] NETs recognized by plasmacytoid dendritic cells induces strong type I IFN and pro-inflammatory cytokine responses in systemic lupus erythematosus (SLE) and autoimmune pancreatitis (AIP).[11–15] Given that human and experimental IBD is caused by type I IFN and pro-inflammatory cytokine responses, PAD4-mediated release of NETs is likely to be involved in the pathogenesis of IBD.[3,16,17] Increased production of NETs has been reported in both human and experimental IBD, suggesting pathogenic roles of PAD2 and/or PAD4-mediated NETs in colonic inflammation.[4,18–23] However, the gene expression mechanisms of *PAD2* and *PAD4* have been poorly understood in human IBD. Here, we provide evidence that the mRNA expression of *PAD2* and *PAD4* is reciprocally regulated in the colonic mucosa of patients with UC, but not in those with CD, and that the expression of the latter PAD isozyme is accompanied by that of IL-6 or TNF-α, but not type I IFNs, in patients with both UC and CD. Thus, this study clarifies the correlation between PAD2/4 and cytokines in patients with IBD.

Materials and Methods

Dextran sodium sulfate (DSS)-induced colitis. Six-week-old female C57BL/6J mice (Japan SLC, Hamamatsu, Japan) were treated with 2% DSS (MW: 36,000–50,000; MP Biomedicals, Santa Ana, CA) in drinking water from days 0 to 5, as previously described.[16,24] Animal experiments were approved by the Review Boards of Kindai University Faculty of Medicine. The mice were also treated with intraperitoneal injection of Cl-amidine (a pan-PAD inhibitor, $n = 14$, 80 mg/kg, Sigma-Aldrich, St. Louis, MO) or dimethyl sulfoxide (DMSO; $n = 14$) as previously reported.[19,25] The animals were sacrificed on day 7, and colonic tissues were stained with hematoxylin and eosin. Scoring of DSS-induced colitis was performed in accordance with previous reports.[16,17]

Isolation of colonic lamina propria mononuclear cells (cLPMNCs). cLPMNCs were isolated using a well-established previously described protocol.[16,17] Briefly, cLPMNCs (1×10^6/ml) were stimulated with PAM_3CSK4 (PAM, 10 μg/ml, InvivoGen, San Diego, CA), lipopolysaccharide (LPS, 1 μg/ml, Sigma-Aldrich), or CpG (1 μM, InvivoGen) for 48 h.[16,17,26,27] To determine IL-6, TNF-α, and IFN-β concentrations, culture

*To whom correspondence should be addressed.
E-mail: tomohiro@med.kindai.ac.jp

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Table 1. Biopsy sites in patients with inflammatory bowel disease

Disease	Ulcerative colitis		Crohn's disease	
Disease activity	Active	Remission	Active	Remission
Ileum	0	0	6	7
Cecum	1	1	0	1
Ascending colon	1	0	0	1
Transverse colon	1	0	1	0
Descending colon	2	3	0	0
Sigmoid colon	3	1	1	0
Rectum	12	15	2	1
Total	20	20	10	10

supernatants were subjected to commercial murine IL-6, TNF-α, and IFN-β enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN).[16,17,26,27]

Patients. Patients with UC and CD were enrolled, as previously described.[16,17] 40 patients with UC (active disease, $n = 20$; remitted disease, $n = 20$) and 20 with CD (active disease, $n = 10$; remitted disease, $n = 10$) were included. The disease activity of UC and CD was determined as previously described, and the patient characteristics have been described in our previous report.[16] Non-tumorous portions of the colonic mucosa in patients with colon adenoma served as healthy colonic mucosa ($n = 4$). Ethical permission for this study was granted by the Review Boards of Kindai University Faculty of Medicine, and written informed consent was obtained from each patient (approval No.: 28-034, KAME-28-028, and KDMS-28-008).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). mRNA was isolated from ileal or colonic biopsy samples obtained from patients with UC and CD during colonoscopies. Biopsy samples were obtained from the ileum, proximal and distal colon, and rectum as indicated in Table 1. The mRNA expression of each target gene was determined using qRT-PCR, as described previously.[16,17,28,29] Briefly, mRNA was isolated from biopsy specimens using TRIzol reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed into cDNA using Superscript III (Invitrogen). SYBR Green-based qPCR was performed using a LightCycler 480 system (Roche, Tokyo, Japan) and Quantitect Primer Assays (Qiagen, Valencia, CA). Each target primer was purchased from Qiagen, and *ACTB* mRNA expression was used as the internal control.

Immunohistochemistry. Deparaffinized colonic sections obtained from patients with active UC ($n = 8$) were fixed in 10% formalin. PAD2 and PAD4 expression was visualized using the DAKO EnVision+ System (DAKO JAPAN, Tokyo, Japan), as previously described, with rabbit human anti-PAD2 antibody (Proteintech, Rosemont, IL) and anti-PAD4 antibody (GeneTex, Irvine, CA).[30]

Statistical analyses. GraphPad Prism (GraphPad Software, San Diego, CA) was used for all statistical analyses.[16,17] The Mann–Whitney *U* test, a nonparametric version of the unpaired *t* test, was used to evaluate the differences between groups. The Kruskal–Wallis test, a nonparametric version of one-way analysis of variance, was used to evaluate the differences between multiple comparisons. For post hoc analysis, the Bonferroni-corrected Mann–Whitney *U* test was performed for comparison between groups. The Pearson's correlation coefficient was calculated in the correlation analyses. Effects were considered significant at $p<0.05$.

Results

Suppression of DSS-induced colitis by Cl-amidine administration. PAD enzymes are indispensable for the generation of citrullinated proteins.[8] Among the five members of PAD isoforms, PAD2 and PAD4 are well studied given their abundant expression in immune cells.[10] NET formation requires citrullination of histones by PAD4, whereas PAD2 activation is involved in NET formation in the absence of PAD4.[9,10] Enhanced production of NETs accompanied by PAD4 activation has been observed in the gut mucosa of patients with active UC and CD.[4] However, the roles played by PAD2/4 have not been fully understood in the context of UC and CD immunopathogenesis.

To clarify roles played by PADs in the development of colitis, we initially examined effects of Cl-amidine, a pan-PAD inhibitor, on the development of DSS colitis. C57BL/6 mice were treated with 2% DSS in drinking water in combination with intraperitoneal injection of Cl-amidine, a pan-PAD inhibitor.[19,25] PAD4 expression was downregulated by Cl-amidine administration in previous studies.[23] Intraperitoneal administration of Cl-amidine inhibited body weight loss caused by treatment with 2% DSS (Fig. 1A). No significant difference in colon length was observed between mice treated with Cl-amidine or DMSO (Cl-amidine vs DMSO, 6.0 ± 0.1 cm vs 5.7 ± 0.1 cm, mean ± SEM). Destruction of crypt architecture and accumulation of immune cells in the colon mucosa were observed in mice treated with 2% DSS and DMSO. In contrast, such pathological findings were barely observed in mice treated with 2% DSS and Cl-amidine. The pathological scores for DSS-induced colitis were significantly lower in mice treated with Cl-amidine than in those treated with DMSO (Fig. 1B and C).

As shown in Fig. 1D, IL-6 and TNF-α production was significantly lower by cLPMNCs from mice treated with Cl-amidine upon stimulation with Toll-like receptor (TLR)2, TLR4, and TLR9 ligands, compared with that in cLPMNCs from mice treated with DMSO. In contrast, the production of IFN-β by cLPMNCs was comparable in mice treated with Cl-amidine or DMSO. These results obtained from DSS-induced colitis suggest that activation of PADs plays a colitogenic role through the production of IL-6 and TNF-α.

PAD2 and PAD4 expression in the colonic mucosa of patients with CD and UC. Having confirmed the involvement of PADs activation in DSS-induced colitis, the mRNA expression of PAD isozymes was examined using colonic biopsy samples. PAD4 expression was markedly higher in the colonic mucosa of active UC patients than in the colonic mucosa of remitted patients and healthy colonic mucosa, although the comparison between healthy colonic mucosa and active UC mucosa did not show significance ($p = 0.0676$; Fig. 2A). A similar tendency was observed in the colonic mucosa of patients with CD, although the difference was not significant. PAD2 expression was significantly lower in patients with active UC than in those with remitted UC and healthy colonic mucosa. In contrast, patients with active and remitted CD exhibited comparable levels of PAD2 expression. *PAD4* mRNA expression was negatively correlated with that of *PAD2* in the colonic mucosa of patients with UC, wherein such a correlation was not observed in patients with CD (Fig. 2B). These data suggest that the expression of PAD4 and PAD2 is positively and negatively correlated with disease activity in UC, respectively. Reciprocal regulation of PAD2 and PAD4 expression is characteristic of UC, but not CD. Consistent with previous reports, PAD4 expression was predominantly observed in neutrophils localized in crypt abscesses and immune cells in the colonic mucosa of active UC patients whereas PAD2 expression was scarcely detected in the same specimens (data not shown).[21,31]

Disease location affects profiles of proinflammatory cytokines in IBD; ileal CD is characterized by T helper type 1 (Th1) or Th17 responses whereas Th1 responses are predominant in colonic CD.[32,33] Subsequently, we sought to determine whether the disease location has an impact on the mRNA expression of PAD2 and PAD4. As shown in Fig. 3A, mRNA expression of

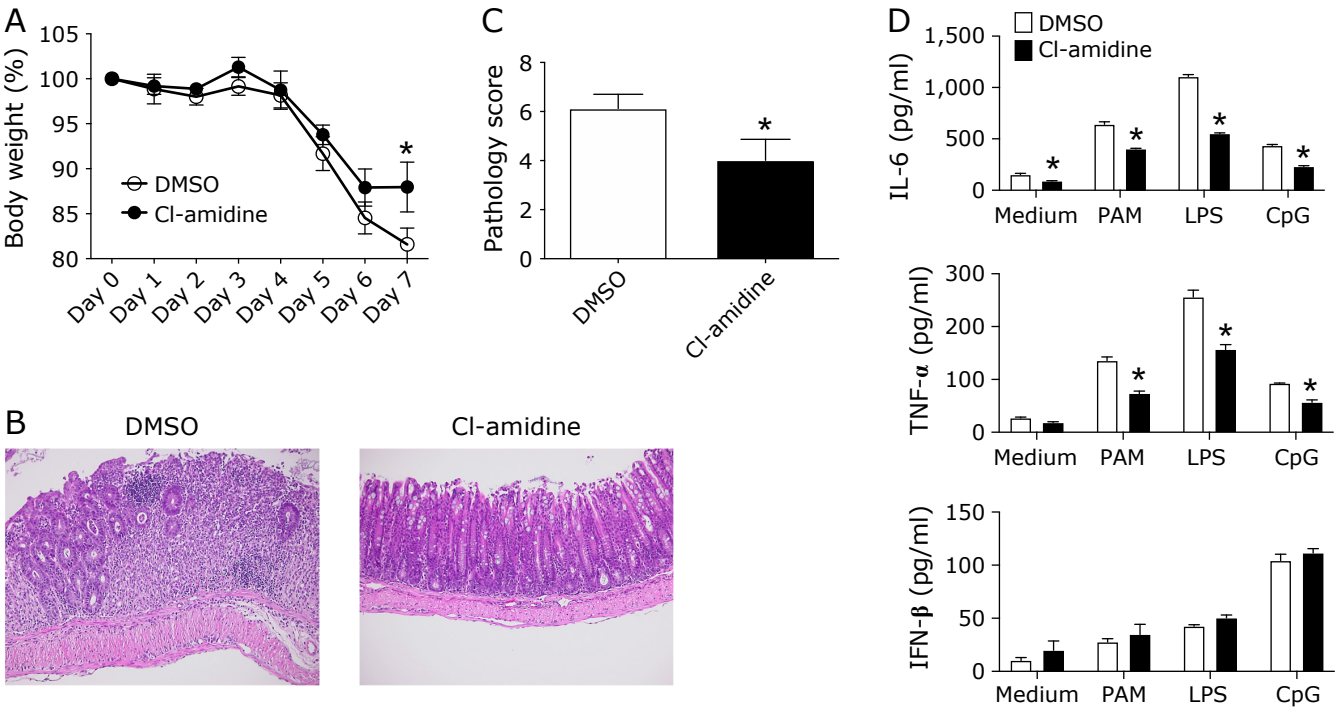

Fig. 1. Suppression of dextran sodium sulfate (DSS)-induced colitis via Cl-amidine administration. C57BL/6 mice were treated with 2% DSS from days 0 to 5; the mice were also treated with an intraperitoneal injection of dimethyl sulfoxide (DMSO, $n = 14$) or Cl-amidine (80 mg/kg, $n = 14$) on days 0 and 5. (A) Changes in body weight. (B, C) Hematoxylin & eosin staining of colonic tissue on day 7; magnification 100×. Pathological scores of DSS colitis on day 7. (D) Colonic lamina propria mononuclear cells (cLPMNCs) were isolated from mice on day 7. cLPMNCs (1×10^6/ml) were stimulated with PAM_3CSK4 (PAM, 10 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), or CpG (1 μM) for 48 h. Culture supernatants were subjected to enzyme-linked immunosorbent assays to determine the concentrations of IL-6, TNF-α, and IFN-β. Data are expressed as mean ± SE. *p<0.05.

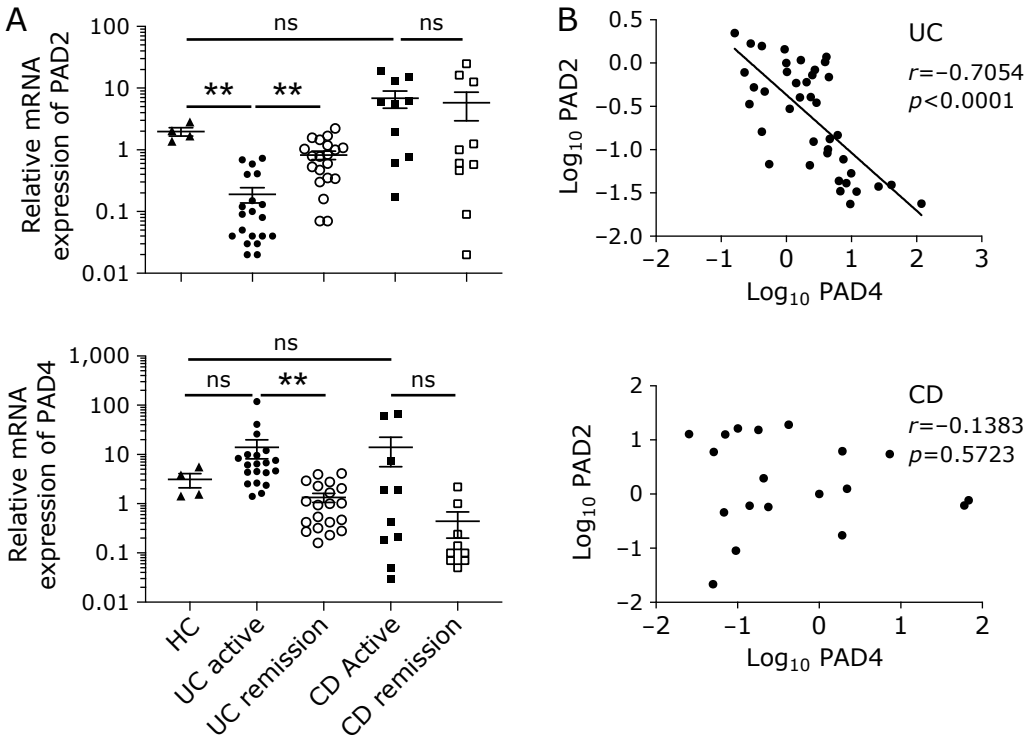

Fig. 2. mRNA expression of protein arginine deiminase 2 and 4 in patients with inflammatory bowel diseases. mRNA was isolated from the ileal and colonic mucosa of patients with ulcerative colitis (UC; active disease, $n = 20$; remitted disease, $n = 20$) and Crohn's disease (CD; active disease, $n = 10$; remitted disease, $n = 10$). Non-tumorous portions of the colonic mucosa in patients with colon adenoma served as healthy colonic mucosa (HC, $n = 4$). qRT-PCR analysis of the mRNA expression levels of protein arginine deiminase (PAD)2 and PAD4. Each dot represents the value for each patient. (A) Data are presented as the mean ± SE. **p<0.01, N.S.; not significant. (B) Correlation between *PAD2* and *PAD4* mRNA expression in patients with UC and CD. *P* values and correlation coefficient (r) values, as determined by Pearson's correlation coefficient, are shown.

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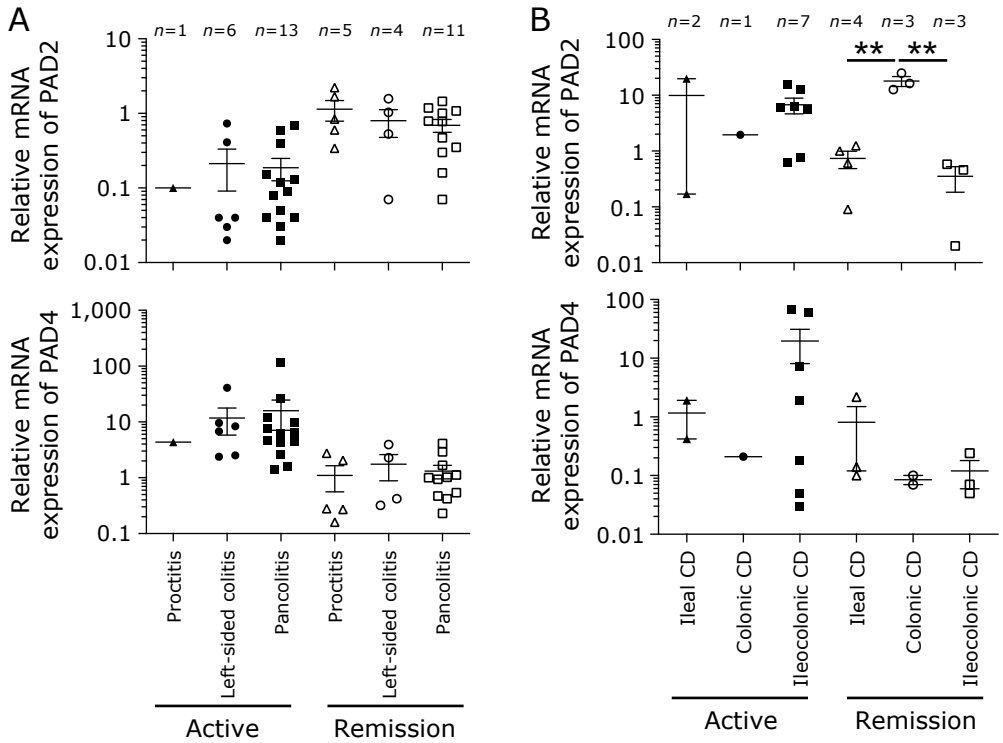

Fig. 3. mRNA expression of protein arginine deiminase 2 and 4 in patients with different types of ulcerative colitis and Crohn's disease. mRNA was isolated from the ileal and colonic mucosa of patients with ulcerative colitis (UC; active disease, $n = 20$; remitted disease, $n = 20$, A) and Crohn's disease (CD; active disease, $n = 10$; remitted disease, $n = 10$, B). qRT-PCR analysis of the mRNA expression levels of protein arginine deiminase (PAD)2 and PAD4. Each dot represents the value for each patient. Data are presented as the mean ± SE. $^{**}p<0.01$.

PAD2 or PAD4 remained unaltered in three different types of UC, i.e., proctitis, left-sided colitis, and pancolitis irrespective of whether the disease was in an active or remission stage. Similarly, no significant alterations in mRNA expression of PAD4 were observed in three different types of CD, i.e., ileal CD, ileocolonic CD, and colonic CD (Fig. 3B). In contrast, mRNA expression of PAD2 was significantly higher in colonic CD than ileal or ileocolonic CD. However, it should be noted that the limited number of patients in each disease type prevents a definitive establishment of the association between mRNA expression of PADs and disease types.

Correlation between PAD2/PAD4 expression and proinflammatory cytokines in the colonic mucosa of patients with UC. Enhanced production of IL-6 and TNF-α underlie the immunopathogenesis of CD and UC.[3] Inhibition of PADs by Cl-amidine suppressed the development of DSS-induced colitis, and these effects were accompanied by reduced IL-6 and TNF-α responses (Fig. 1). In human samples, we observed reciprocal regulation of *PAD2* and *PAD4* mRNA expression in UC. These data obtained in both experimental and human IBD prompted us to examine the relationship between PAD2/4 expression and proinflammatory cytokine responses. Given that PAD4-mediated NET formation is a strong inducer for production of type I IFNs, we initially focused on the expression of IFN-stimulated genes (ISGs) and prototypical proinflammatory cytokines.[11–14] No significant correlation was observed between the mRNA expression of *PAD4* and type I IFNs or ISGs, including TNF receptor-associated factor 3 (*TRAF3*), interferon regulatory factor 3 (*IRF3*), and deubiquitinating enzyme A (*DUBA*, data not shown), whereas *PAD4* expression was parallel to that of C-X-C motif chemokine ligand 10 (*CXCL10*) and *IRF7* (Fig. 4A). Thus, PAD4 expression is unlikely to be involved in type I IFN-mediated signaling pathways in patients with UC. CXCL8 is a chemoattractant for neutrophils expressing PAD4.[24] As expected, strong positive correlation was noted between the expression of CXCL8 and PAD4. In addition, a positive correlation between the expression of PAD4 and IL-6 or TNF-α, but not IL-12/23p40, was observed in patients with UC. These data suggest that the mRNA expression of *PAD4* was positively correlated with that of the prototypical colitogenic mediators, *CXCL8*, *IL-6*, and *TNF-α*.

Consistent with the strong negative correlation between the mRNA expression of *PAD2* and *PAD4*, *PAD2* expression was negatively correlated to that of *CXCL8*, *CXCL10*, or *IL*-6 in the colonic mucosa of patients with UC (Fig. 4B). A positive weak correlation was observed between the mRNA expression of *IFN-α4* and *PAD2* in these patients. These data suggest that the activation of PAD4, but not PAD2, is involved in the colitogenic cytokine and chemokine responses in UC.

Correlation between PAD2/PAD4 expression and proinflammatory cytokines in the colonic mucosa of patients with CD. As in the case of patients with UC, the mRNA expression of *PAD4* was not correlated with that of *IFN-α4*, *IFN-β*, *IL-12/23p40*, *TRAF3*, *DUBA* (data not shown), *IRF3*, and *IRF7*, suggesting that PAD4 is not involved in type I IFN-mediated signaling pathways (Fig. 5A). In contrast, a positive correlation was observed between the mRNA expression of *PAD4* and that of *CXCL8*, *CXCL10*, *IL-6*, and *TNF-α*. The mRNA expression of *PAD2* was not correlated with that of *IFN-α4*, *IFN-β*, *CXCL8*, *CXCL10*, *IL-6*, and *TNF-α* (Fig. 5B). Collectively, these data suggest a positive correlation between the mRNA expression of *PAD4* and that of *IL-6*, *TNF-α*, *CXCL8*, and *CXCL10* in the colonic mucosa of patients with UC and CD.

Discussion

Activation of PAD2 and PAD4 is involved in the production of proinflammatory cytokines induced by NETs.[8–10] Although proinflammatory cytokine responses have been implicated in the

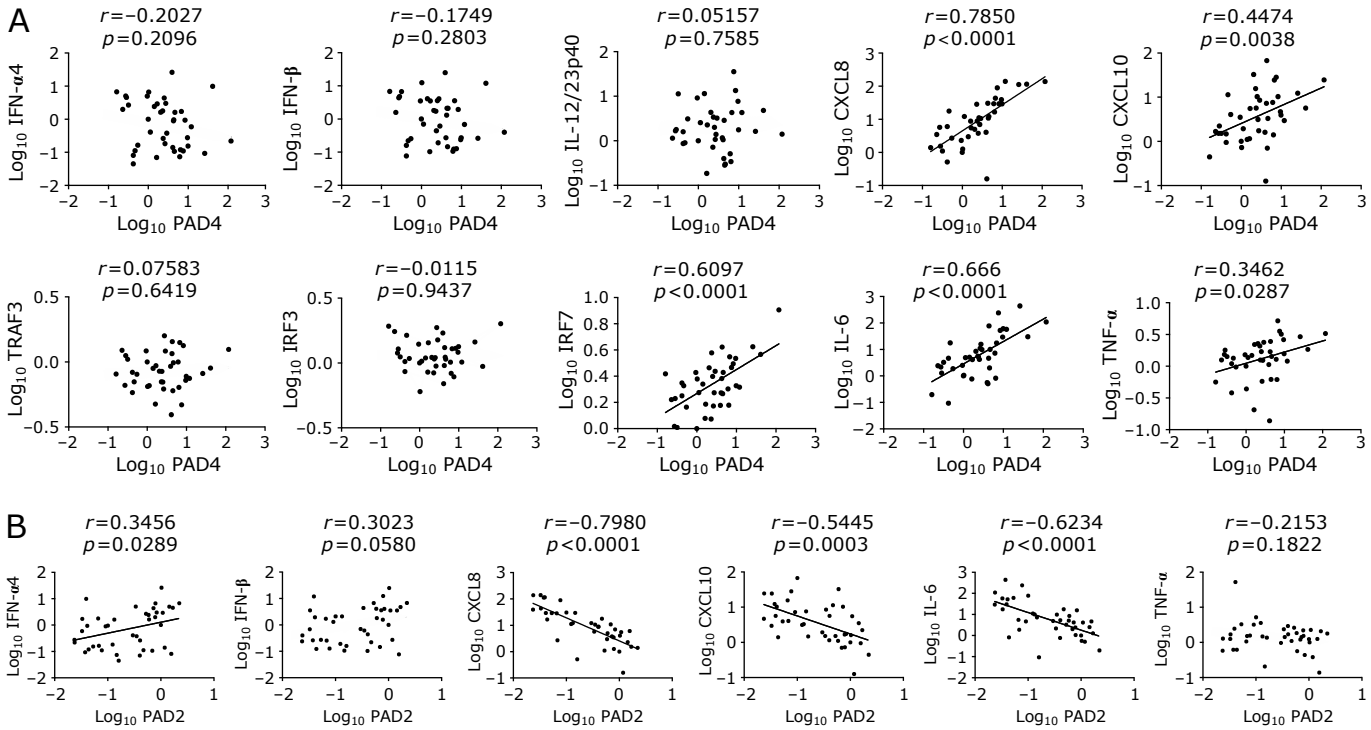

Fig. 4. Correlation between the mRNA expression of protein arginine deiminase 4 (*PAD4*) and cytokines in patients with ulcerative colitis (UC). mRNA was isolated from the colonic mucosa of patients with UC. (A) Correlation between the mRNA expression of *PAD4* and *IFN-α4*, *IFN-β*, *IL12/23p40*, C-X-C motif chemokine ligand 8 (*CXCL8*), *CXCL10*, TNF receptor-associated factor 3 (*TRAF3*), interferon regulatory factor 3 (*IRF3*), *IRF7*, *IL-6*, and *TNF-α*. (B) Correlation between the mRNA expression of *PAD2* and *IFN-α4*, *IFN-β*, *CXCL8*, *CXCL10*, *IL-6*, and *TNF-α*. Each dot represents the value for each patient. *P* values and correlation coefficient (*r*) values, as determined by Pearson's correlation coefficient, are shown.

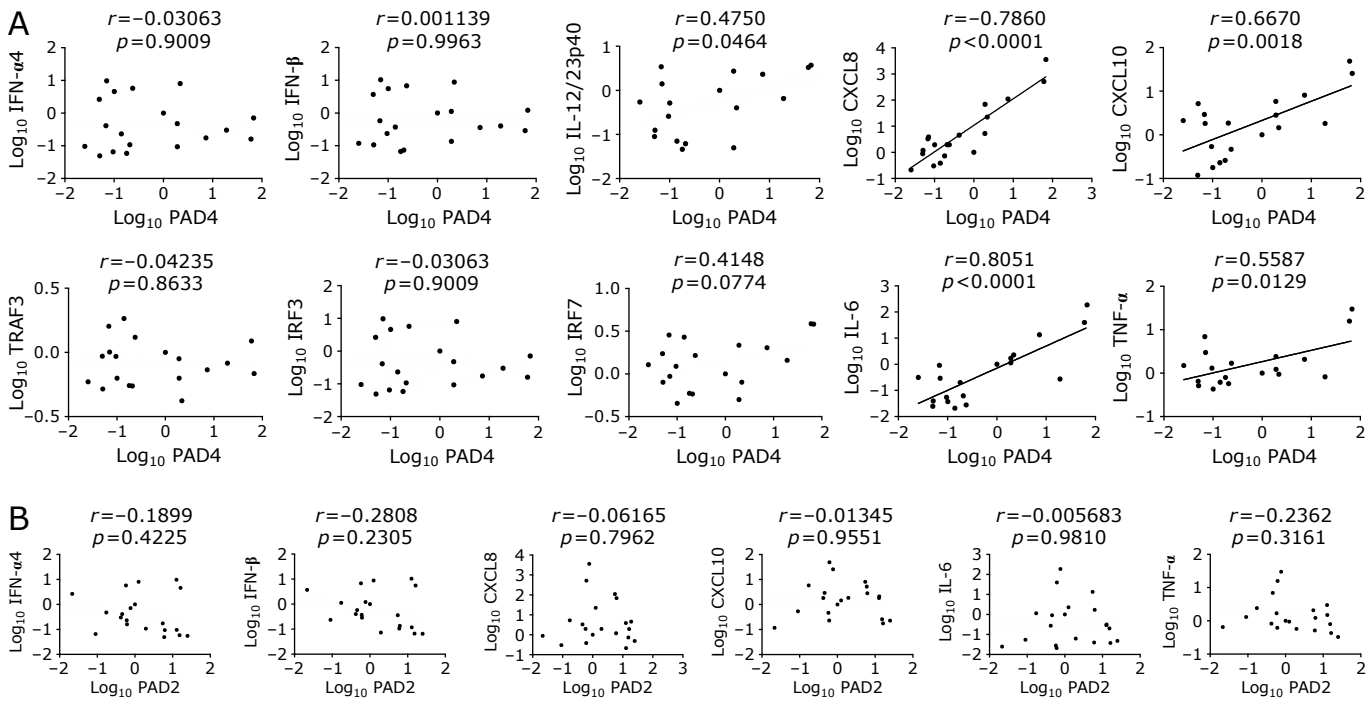

Fig. 5. Correlation between the mRNA expression of protein arginine deiminase 4 (*PAD4*) and cytokines in patients with Crohn's disease (CD). mRNA was isolated from the ileal and colonic mucosa of patients with CD. (A) Correlation between the mRNA expression of *PAD4* and *IFN-α4*, *IFN-β*, *IL12/23p40*, C-X-C motif chemokine ligand 8 (*CXCL8*), *CXCL10*, TNF receptor-associated factor 3 (*TRAF3*), interferon regulatory factor 3 (*IRF3*), *IRF7*, *IL-6*, and *TNF-α*. (B) Correlation between the mRNA expression of *PAD2* and *IFN-α4*, *IFN-β*, *CXCL8*, *CXCL10*, *IL-6*, and *TNF-α*. Each dot represents the value for each patient. *P* values and correlation coefficient (*r*) values, as determined by Pearson's correlation coefficient, are shown.

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immunopathogenesis of IBD,[3,16,17] the relationship between the expression of PADs and proinflammatory mediators is poorly understood. In this study, we explored the mRNA expression of *PAD2* and *PAD4* in the gut mucosa of patients with CD and UC. Consistent with previous reports, we found that *PAD4* expression was markedly higher in the colonic mucosa of patients with active UC than in those with remitted UC.[4,21,31] In contrast, induction of remission was accompanied by a significant increase in *PAD2* expression. The negative correlation between *PAD2* and *PAD4* mRNA expression in the colonic mucosa also indicates that PAD2 and PAD4 expression is negatively and positively correlated with disease activity in UC, respectively. The mRNA expression of *PAD4* was higher in the gut mucosa of patients with active CD than in remitted patients, although the difference was not significant. Our mRNA expression analyses using samples from patients with IBD show the reciprocal regulation of PAD2 and PAD4 expression in the gut mucosa of UC patients, but not CD patients, suggesting that PAD2 and PAD4 activation is associated with the suppression and exacerbation of colonic inflammation, respectively, in patients with UC. This concept is further supported by our observation that colonic expression of proinflammatory mediators is positively and negatively correlated to that of PAD4 and PAD2 in UC, respectively.

A positive correlation between PAD4 and CXCL8, IL-6, or TNF-α was consistently observed in mRNA expression analyses for both UC and CD (Fig. 4 and 5). Considering that CXCL8 is a strong chemoattractant for neutrophils producing NETs, these data suggest involvement of CXCL8-NETs-TNF-α axis in the immunopathogenesis of UC and CD, as suggested by previous reports.[4,18–24] In contrast, PAD2 mRNA expression exhibited a negative correlation with CXCL8 and IL-6 in UC, but not in CD. Presently, it remains unknown why the negative correlation between PAD2 and proinflammatory mediators is specific to UC. In this regard, not only neutrophils but also monocytes express PAD2.[10] Thus, differences in immune cell composition between UC and CD at the remission phases might have affected mRNA expression of PAD2. Alternatively, mRNA expression of PAD2 might be associated with disease location because PAD2 mRNA was significantly higher in colonic CD than in ileal CD at the remission phase (Fig. 3B). In addition, we could not exclude the possibility that treatment with biologics might have affected mRNA expression of PADs. In this study, 80% and 27.5% of patients with CD and UC were treated with biologics, respectively.

Administration of Cl-amidine, a pan-PAD inhibitor, suppressed the development of DSS-induced colitis, which was accompanied by the reduced production of IL-6 and TNF-α by cLPMNCs upon exposure to TLR ligands. Importantly, the production of IFN-β by cLPMNCs was comparable in mice treated with DMSO or Cl-amidine. Thus, inhibition of PADs by Cl-amidine prevented experimental colitis by suppressing proinflammatory cytokine production (IL-6 and TNF-α), but not that of type I IFNs mediated by TLRs. Together with the correlation between the mRNA expression of *PAD4* and *IL-6* or *TNF-α* in human IBD, these results suggest that PAD4 activation is involved in chronic colitis through signaling pathways mediated by IL-6 and TNF-α. Consistent with these results, blockade of PAD activation by Cl-amidine has been shown to attenuate experimental colitis.[19,23,25] However, caution needs to be exercised regarding the interpretation of animal colitis data since Cl-amidine can inhibit not only PAD4 but also other PADs.[34,35] Therefore, it is possible that amelioration of experimental colitis by Cl-amidine might be mediated by inhibition of other PADs rather than PAD4, despite a marked reduction in the expression of PAD4 and citrullinated proteins in the colonic mucosa.[19,23,25]

PAD4-dependent NET release plays a pathogenic role in the development of SLE and AIP.[11–14,36] Excessive production of PAD4-dependent NETs underlies the immunopathogenesis of SLE and AIP through its strong induction of type I IFN and ISG responses.[11–15] Enhanced expression of ISGs has been observed in the active colonic mucosa of patients with UC and CD.[16,37] Recent studies have provided evidence that NET release is involved in sustained inflammation in both experimental and human IBD.[4,18–23,38] Considering that strong type I IFN responses upon exposure to NETs are implicated in SLE and AIP, we compared the mRNA expression levels of *PAD2* and *PAD4* with those of *IFN-α4*, *IFN-β*, and ISGs. We found no significant correlation between *PAD2/PAD4* expression and that of type I IFNs or ISGs, except for *CXCL10* and *IRF7*, in the colonic mucosa of patients with UC or CD. In contrast, the colonic mRNA expression levels of *PAD4* were parallel to those of the prototypical proinflammatory mediators, *IL-6* and *TNF-α*, in both patients with UC and CD. Positive correlation between *PAD4* and *CXCL10* or *IRF7* expression can be explained by promoter analyses, which show that CXCL10 and IRF7 promoter regions contain nuclear factor-κB (NF-κB)-binding consensus sequences induced by TNF-α.[39,40] Thus, it is likely that PAD4-mediated NETs induce colonic inflammation through the activation of signaling pathways mediated by IL-6 and TNF-α, rather than type I IFNs. In line with this notion, cLPMNCs isolated from patients with UC produce large amounts of TNF-α and IL-1β upon stimulation with NETs.[18,21] However, we cannot completely exclude the possibility of protective rather than pathogenic roles played by PAD4-mediated NETs in experimental and human IBD. Leppkes *et al.*[31] showed that immunothrombosis formation by PAD4 activation contributes to the maintenance of intestinal homeostasis by prevention of rectal bleeding and thereby inhibits the exacerbation of UC. Alternatively, aggregated NETs contribute to the resolution of inflammation by degrading cytokines and chemokines via serine proteases.[41] Pathogenic or beneficial roles played by PAD4 need to be determined in future studies.

As for molecular mechanisms accounting for the positive correlation between the mRNA expression of *PAD4* and *IL-6* or *TNF-α*, we considered the involvement of nuclear PAD4 as a transcriptional regulator.[8] Nuclear PAD4 directly citrullinates the NF-κB subunit p65 to enhance TNF-α transcription in neutrophils.[42] Importantly, TNF-α production by neutrophils upon exposure to LPS is significantly reduced by Cl-amidine through downregulation of nuclear translocation of p65.[42] Thus, PAD4's nuclear localization enables it to enhance the transcription of p65-dependent IL-6 and TNF-α.[42] Our data regarding cytokine profiles in experimental models and humans with IBD fully support the concept that citrullination activity of nuclear PAD4 mediates colitis through induction of IL-6 and TNF-α, but not type I IFN responses. Additionally, the predominant cytosolic localization of PAD2 may be associated with the lack of positive correlation between pro-inflammatory cytokine responses and this PAD isozyme.

In conclusion, we found that the mRNA expression of *PAD2* and *PAD4* is reciprocally regulated in the colonic mucosa of patients with UC and that their levels are negatively and positively correlated with UC disease activity, respectively. Colonic *PAD4* mRNA expression was parallel to that of *IL-6* and *TNF-α* in both patients with UC and CD. The mRNA expression of PAD2 and PAD4 holds promise as potential biomarkers for UC, and inhibiting the latter PAD isozyme may prove beneficial for treating patients with IBD. However, the confirmation of this hypothesis awaits further studies that comprehensively address the mRNA expression of PAD2 and PAD4 in both active and remitted mucosa of the small and large intestine. These investigations need to involve a large number of patients, encompassing a diverse cohort of patients with UC and CD.

Author Contributions

Conceptualization: YO, YM, KM, and TW; Methodology: YO,

YM, KM, and TW; Formal analysis and investigation: YO, YM, KM, NO, AH, RT, SM, KK, HH, and TW; Writing—original draft preparation: YO, KY, and TW; Writing—review and editing: YO, KM, KY, and TW; Funding acquisition: TW; Resources: SM and HH; Supervision: MK.

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Data Availability Statement

The data that support the findings of this study are available upon reasonable request from the corresponding author.

Ethics Approval Statement

Ethical permission for this study was granted by the Review Boards of Kindai University Faculty of Medicine (approval No.: 28-034, KAME-28-028, and KDMS-28-008).

Abbreviations

AIP	autoimmune pancreatitis
CD	Crohn's disease
cLPMNs	colonic lamina propria mononuclear cell
CXCL	C-X-C motif chemokine ligand
DMSO	dimethyl sulfoxide
DSS	dextran sodium sulfate
DUBA	deubiquitinating enzyme A
IBD	inflammatory bowel disease
IRF	interferon regulatory factor
ISG	IFN-stimulated gene
LPS	lipopolysaccharide
NET	neutrophil extracellular trap
NF-κB	nuclear factor-κB
PAD	protein arginine deiminase
PAM	PAM_3CSK4
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
Th1	T helper type 1
TLR	Toll-like receptor
TRAF3	TNF receptor-associated factor 3
UC	ulcerative colitis

Conflict of Interest

No potential conflicts of interest were disclosed.

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