13. Leffler DA, Lamont JT: Treatment of *Clostridium difficile*-associated disease. Gastroenterology 2009; **136**: 1899–1912. [Medline] [CrossRef]
14. Kelly CR, de Leon L, Jasutkar N: Fecal microbiota transplantation for relapsing *Clostridium difficile* infection in 26 patients: methodology and results. J Clin Gastroenterol 2012; **46**: 145–149. [Medline] [CrossRef]
15. Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, McFarland LV, Mellow M, Zuckerbraun BS: Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. Am J Gastroenterol 2013; **108**: 478–498, quiz 499. [Medline] [CrossRef]
16. Kelly CR, Kunde SS, Khoruts A: Guidance on preparing an investigational new drug application for fecal microbiota transplantation studies. Clin Gastroenterol Hepatol 2014; **12**: 283–288. [Medline] [CrossRef]
17. Sartor RB: Microbial influences in inflammatory bowel diseases. Gastroenterology 2008; **134**: 577–594. [Medline] [CrossRef]
18. Takaishi H, Matsuki T, Nakazawa A, Takada T, Kado S, Asahara T, Kamada N, Sakuraba A, Yajima T, Higuchi H, Inoue N, Ogata H, Iwao Y, Nomoto K, Tanaka R, Hibi T: Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. Int J Med Microbiol 2008; **298**: 463–472. [Medline] [CrossRef]
19. Ott SJ, Plamondon S, Hart A, Begun A, Rehman A, Kamm MA, Schreiber S: Dynamics of the mucosa-associated flora in ulcerative colitis patients during remission and clinical relapse. J Clin Microbiol 2008; **46**: 3510–3513. [Medline] [CrossRef]
20. Bennet JD, Brinkman M: Treatment of ulcerative colitis by implantation of normal colonic flora. Lancet 1989; **1**: 164. [Medline] [CrossRef]
21. Borody TJ, Warren EF, Leis S, Surace R, Ashman O: Treatment of ulcerative colitis using fecal bacteriotherapy. J Clin Gastroenterol 2003; **37**: 42–47. [Medline] [CrossRef]
22. Kump PK, Gröchenig HP, Lackner S, Trajanoski S, Reicht G, Hoffmann KM, Deutschmann A, Wenzl HH, Petritsch W, Krejs GJ, Gorkiewicz G, Högenauer C: Alteration of intestinal dysbiosis by fecal microbiota transplantation does not induce remission in patients with chronic active ulcerative colitis. Inflamm Bowel Dis 2013; **19**: 2155–2165. [Medline] [CrossRef]
23. Kunde S, Pham A, Bonczyk S, Crumb T, Duba M, Conrad H, Cloney D, Kugathasan S: Safety, tolerability, and clinical response after fecal transplantation in children and young adults with ulcerative colitis. J Pediatr Gastroenterol Nutr 2013; **56**: 597–601. [Medline] [CrossRef]
24. Quera R, Espinoza R, Estay C, Rivera D: Bacteremia as an adverse event of fecal microbiota transplantation in a patient with Crohn's disease and recurrent *Clostridium difficile* infection. J Crohn's Colitis 2014; **8**: 252–253. [Medline] [CrossRef]
25. Gordon H, Harbord M: A patient with severe Crohn's colitis responds to faecal microbiota transplantation. J Crohn's Colitis 2014; **8**: 256–257. [Medline] [CrossRef]
26. Borody TJ, Paramsothy S, Agrawal G: Fecal microbiota transplantation: indications, methods, evidence, and future directions. Curr Gastroenterol Rep 2013; **15**: 337. [Medline] [CrossRef]
27. Jeffery IB, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M: An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 2012; **61**: 997–1006. [Medline] [CrossRef]
28. Vrieze A, Van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JF, Dallinga-Thie GM, Ackermans MT, Serlie MJ, Oozeer R, Derrien M, Druesne A, Van Hylckama Vlieg JE, Bloks VW, Groen AK, Heilig HG, Zoetendal EG, Stroes ES, de Vos WM, Hoekstra JB, Nieuwdorp M: Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 2012; **143**: 913–916, e7. [Medline] [CrossRef]
29. Udayappan SD, Hartstra AV, Dallinga-Thie GM, Nieuwdorp M: Intestinal microbiota and faecal transplantation as treatment modality for insulin resistance and type 2 diabetes mellitus. Clin Exp Immunol 2014; **177**: 24–29. [Medline] [CrossRef]

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Regulation of anergy-related ubiquitin E3 ligase, GRAIL, in murine models of colitis and patients with Crohn's disease

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Abstract
Background Abrogating tolerance is a critical step in the pathogenesis of Crohn's disease (CD). T cell-anergy is one of the main mechanisms of tolerance and is regulated by the gene related to anergy in lymphocytes (GRAIL). This study investigated the expressions and regulation of GRAIL in CD and murine colitis models.
Methods Expressions of GRAIL mRNA and protein in $CD4^+$ T cells were investigated in the peripheral blood and mucosal tissues of patients with CD, mice with dextran sodium salt (DSS)-induced colitis, and *Il-10*-deficient mice. MicroRNAs responsible for the regulation of GRAIL were examined by miRNA microarray. GRAIL-overexpressing T cells were intravenously injected in mice with DSS-induced colitis.
Results The GRAIL expression was higher in the lamina propria (LP) $CD4^+$ T cells of CD patients than of the control subjects, while it was lower in the peripheral blood $CD4^+$ T cells of the CD patients than of the control subjects. The GRAIL mRNA expression was lower, but the GRAIL protein expression was higher in the LP of colitic mice than that of non-colitic mice. The miRNA microarray identified miR-290-5p as an miRNA that inhibits expression of the GRAIL protein and that is highly expressed in the LP of non-colitic mice. GRAIL-expressing T cells expressed regulatory T cell markers and showed suppressive effects in murine DSS-induced colitis.
Conclusions Our results show that expression of GRAIL is uniquely regulated by the specific miRNA in the intestinal mucosa, and suggest that GRAIL may associate with the pathophysiology of CD.

Keywords Inflammatory bowel disease · MicroRNA · GRAIL · Regulatory T cells

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Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two major categories of inflammatory bowel diseases (IBD) in the human gastrointestinal tract. Both genetic susceptibility and dysregulation of mucosal immune responses against enteric host flora have pivotal roles in mucosal injury [1–3], and abrogating tolerance against unidentified antigens is a critical step in the pathogenesis

of IBD [4]. Clonal anergy of T cells is one of the main mechanisms of tolerance [5], and failure to induce clonal anergy is considered to be associated with the pathogenesis of IBD [6, 7]. T-cell anergy is tightly regulated by E3 ubiquitin ligases, including the gene related to anergy in lymphocyte (GRAIL), the casitas B lineage lymphoma (cbl) family, itchy homologue E3 ubiquitin protein ligase (Itch), and the neural precursor cell expressed developmentally downregulated 4 (NEDD4) [8]. Among these, the role of GRAIL in T-cell anergy has been most vigorously investigated. GRAIL is a type I transmembrane protein that localizes to the endocytic pathway and bears homology to the 'really interesting new gene' (RING) zinc-finger proteins [8]. GRAIL has been shown to be expressed in anergic $CD4^+$ T cells [5, 8]. The expression of GRAIL in $CD4^+$ T cells induces T-cell anergy by limiting interleukin (IL)-2 and IL-4 production. When DO11.10 T cells were infected with an ecotropic retrovirus constitutively expressing wild-type GRAIL, their proliferative capacity in response to antigen and antigen-presenting cells was diminished. Recent studies revealed that GRAIL inhibits the T cell activation cascade by the ubiquitination of CD40 ligand (CD40L), CD83, and T cell receptor (TCR)-CD3 [9–11]. In addition to the importance of GRAIL in converting T cells to an anergic phenotype, a recent study showed that the forced expression of GRAIL in DO11.10 T cells was sufficient for the conversion of these cells to a regulatory phenotype [12]. Additionally, in a Staphylococcal enterotoxin B (SEB)-mediated model of T cell unresponsiveness in vivo, the SEB-exposed forkhead box P3 $(FoxP3)^+GRAIL^+$ T cells were shown to be highly suppressive and non-proliferative, independent of CD25 expression level and glucocorticoid-induced, tumor necrosis factor receptor-related protein (GITR) [13, 14]. This model system revealed a novel paradigm for chronic, non-canonical TCR engagement leading to the development of highly suppressive $FoxP3^+GRAIL^+CD4^+$ T cells. In fact, GRAIL-deficient ($grail^{-/-}$) mice exhibit a susceptibility to autoimmune disease [11]. The importance of GRAIL in human diseases was further demonstrated by our recent study showing that GRAIL expression was increased in the peripheral blood $CD4^+$ T cells of UC patients in remission compared to those of healthy subjects and active UC patients [15]. In addition, GRAIL expression was increased after the effective treatments for active UC patients. To date, no previous reports have investigated the expression of GRAIL in patients with CD and animal models of IBD. In addition, GRAIL expression has not been investigated in the intestine, which comprises the largest pool of immune cells in the human body [16]. We demonstrate here for the first time the expression of GRAIL in patients with CD and in murine colitis models. We also present a novel mechanism in terms of the regulation of GRAIL in the intestine by a specific microRNA (miRNA).

Materials and methods

Human samples

Blood samples were obtained from CD patients who were hospitalized in Osaka University Hospital or from healthy volunteers (HV) recruited in Osaka University Hospital. Intestinal tissues were obtained from the patients with CD or patients with colon cancer who were subjected to surgical resection of the small intestine or the colon in Osaka University Hospital. Patients were diagnosed as having CD according to the endoscopic, radiologic, histological, and clinical criteria provided by the International Organization for the Study of Inflammatory Bowel Disease [17, 18]. The disease activity of CD was evaluated by the Crohn's disease activity index (CDAI) [19]. A CDAI above 150 was defined as active, whereas a CDAI $\leq$150 was defined as remission.

Mice

C57BL/6J mice were purchased from SLC (Shizuoka, Japan). *Il-10*$^{-/-}$ mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate dextran sodium sulfate (DSS) colitis, we orally administered 2 % DSS to female C57BL/6J mice (age 8–12 weeks) in their drinking water for 7 days. All mice were kept under specific pathogen-free conditions in an environmentally-controlled, clean room at the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. The spleen (SP) and mesenteric lymph node (MLN) were aseptically extracted, and single-cell suspensions were prepared by a standard mechanical disruption procedure [20, 21]. Mononuclear cells in the intestinal lamina propria (LP) were prepared by an enzymatic dissociation, as described previously [20, 21]. Briefly, after Peyer's patches were removed from the intestine, the epithelial cell layers were removed from intestinal tissue by incubation in RPMI1640 (Sigma Aldrich, St. Louis, MO, USA) containing ethylenediamine tetraacetic acid (EDTA) [20]. The specimens were dissociated in RPMI1640 containing collagenase (Wako, Osaka, Japan) by stirring at 37 °C. Mononuclear cells were isolated using the discontinuous density gradients procedure with Percoll (GE Healthcare, Pewaukee, WI, USA).

Quantitative real-time RT-PCR

$CD4^+$ T cells from human peripheral blood mononuclear cells (PBMCs) or murine lymphoid tissues were isolated by magnetic cell separation using anti-CD4 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was obtained by reverse-transcription of mRNA with High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). The miRNA fraction was prepared using an miRNA Isolation kit (Ambion, Austin, TX, USA), and 10 ng of total RNA were reverse-transcribed by a MicroRNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analyses for GRAIL (human ID, Hs00226053; mouse ID, Mm00506597), FoxP3 (ID, Mm00475162), GITR (ID, Mm00437136), CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4; ID, Mm00486849), β-actin, miR-290-5p (ID, 002590), and snoRNA135 (ID, 001234) were performed using ready-to-use assays in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). β-actin and snoRNA135 were used as the endogenous controls.

Immunoblot analysis

Human $CD4^+$ T cells from PBMC or murine $CD4^+$ T cells were lysed in radio-immunoprecipitation assay (RIPA) buffer. Protein samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane, as previously described [15]. The membrane was probed with rabbit anti-human GRAIL antibody (IMGENEX, San Diego, CA), rat anti-mouse GRAIL antibody (BD Biosciences, Franklin Lakes, NJ, USA) or rabbit anti-β-actin antibody (Cell Signaling Technology, Danvers, MA, USA). The blots were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Immunohistochemistry

Paraffin-embedded tissue sections were subjected to antigen retrieval by incubation in a pressurized heating chamber (Dako, Carpinteris, CA, USA). Tissue sections were reacted in combination with rat anti-mouse GRAIL antibody (BD Biosciences), rabbit anti-human GRAIL antibody (IMGENEX, San Diego, CA, USA), mouse anti-human CD4 antibody (Abcam, Cambridge, UK), or mouse anti-human FoxP3 antibody (Abcam) as first antibodies, followed by Alexa Flour 488®-labeled anti-rat or anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) or Texas Red-labeled anti-mouse IgG antibody (Abcam) as a secondary antibody. Nuclear staining was performed by DAPI (4′,6-diamidino-2-phenylindole). These stained samples were examined using fluorescence microscopy (Keyence, Osaka, Japan) or three-dimensional, structured illumination microscopy (Carl Zeiss, Oberkochen, Germany). In mice transferred with $GRAIL^{high}$ DO11.10 cells, the tissue sections were stained with anti-mouse GFP antibody (Clontech), and color was developed by 3′-diaminobenzidine using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA).

miRNA microarray analysis

For the miRNA microarray analysis, 0.5 μg of total RNA from $CD4^+$ T cells of the SP or LP were labeled using a miRCURY LNA™ microRNA Array Power Labeling kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Each sample of RNA labeled with Hy5 was hybridized with a highly sensitive DNA chip, *3D-Gene*™ (Toray Industries, Tokyo, Japan) at 37 °C for 16 h. Hybridization signals derived from Hy5 were scanned using a 3D-Gene Scanner (Toray). The scanned image was analyzed and scaled by global normalization [22]. All microarray experiments were performed in compliance with MIAME (Minimum Information About a Microarray Experiment) guidelines [23].

miRNA target predictions

miRgator (http://mirgator.kobic.re.kr:8080/MEXWebApp/), miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi), and MicroCosmTargets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) were used to predict miRNAs that potentially combine with the 3′-untranslated region (3′UTR) of GRAIL mRNA.

Transfection

DO11.10 cells (a murine T cell line, purchased from European Collection of Cell Cultures, Salisbury, UK) were transfected with anti-miR-290-5p inhibitor (50 nM, Ambion) by electroporation (250 V, 950 μF). Anti-miRNA Inhibitors Negative Control #1 (Ambion) was used as endogenous control. Seventy-two hours after the transfection of anti-miR-290-5p inhibitor or negative control #1, cells were collected, and cellular lysates were subjected to western blot using an anti-GRAIL antibody. The GRAIL fragment was inserted into a pAcGFP vector (Clontech, Palo Alto, CA, USA), and the pAcGFP-GRAIL vector or empty vector was transfected into DO11.10 cells by X-tremeGENE HP (Roche, Basel, Switzerland) according to the manufacturer's instructions. $EGFP^+$ DO11.10 cells ($2–5 \times 10^4$ cells/body) sorted by flow cytometry (FACSAria™ II) were intravenously injected into

C57BL/6J mice 2 days after the 2 % DSS administration. The body weight, and the length and histology of the colon were evaluated on day 9.

Luciferase assay

Synthesized oligonucleotides of GRAIL-3′UTR construct, which contained putative miR-290-5p binding sites, were inserted downstream of Luciferase in a pMIR-REPORT Luciferase vector (Ambion). PGL4 *Renilla* Luciferase vector (Promega, Madison, WI, USA) was used to compensate for the transfection efficacy. These constructs and pre-miR 290 precursors (40 nM) or pre-miR^{TM} miRNA negative controls #1 (Ambion) were co-transfected into NIH-3T3 (murine fibroblast cell line) using Lipofectamine 2000 (Invitrogen). Firefly luciferase activity was measured 48 h after transfection using a Dual-Glo^{TM} Luciferase Assay System (Promega). The firefly luciferase activity was evaluated after the compensation by *Renilla* luciferase activity.

Histological analysis

Histological scores for DSS colitis were determined, as previously described, as the sum of the following scores [24]: changes in the epithelium (0, normal; 1, focal loss of goblet cells; 2, diffuse loss of goblet cells; 3, focal loss of crypts; 4, diffuse loss of crypts) and for cell infiltration (0, no increase; 1, around bases of the crypts; 2, diffuse infiltration along the layer of muscularis mucosae; 3, complete infiltration of the mucosal layer; 4, infiltration of both mucosal and submucosal layers).

Cytokine ELISA

DO11.10 cells were transfected with pAcGFP-GRAIL or an empty vector as control, and AcGFP$^+$ T cells were sorted using flow cytometry 48 h after the transfection. Culture supernatants of the cells were collected after activation with plate-coated, anti-CD3 antibody (5 μg/ml) and soluble, anti-CD28 antibody (5 μg/ml), and were analyzed by IL-10, TGF-β, and IL-2 ELISA kit according to manufacturer's instructions (eBioscience, San Diego, CA, USA).

Statistical methods

Student's *t* test, the Wilcoxon signed-rank test and the Tukey HSD test were used for statistical analysis. The body weight of mice injected with DO11.10 was evaluated by a two-factor, repeated ANOVA. $P < 0.05$ was considered statically significant and data were presented as the mean + SEM.

Ethical considerations

The Institutional Review Board of Osaka University Hospital approved the human study protocol, and written informed consent was obtained from each participant. The Institutional Animal Committee on Animal Research approved the mouse protocol.

Results

Expression of GRAIL mRNA and protein in CD4$^+$ T cells in the peripheral blood of the patients with CD

We examined the expression of GRAIL in the peripheral blood of 37 patients with CD and 22 healthy volunteers (HV). The characteristics of the subjects are listed in Table 1. The GRAIL mRNA levels in the CD4$^+$ T cells were significantly lower in the peripheral blood of the patients with active CD than in the HV (Fig. 1a). In addition, the GRAIL mRNA levels were significantly lower even in the CD patients in remission than in the HV (Fig. 1a). Consistent with the lower GRAIL mRNA expression in CD, the protein levels of GRAIL were lower in the peripheral blood CD4$^+$ T cells of the CD patients than in those of the HV (Fig. 1b). These results were different from our previous results of UC patients, where GRAIL mRNA in the remissive UC patients was higher than in healthy volunteers and the expressions of active UC patients and healthy volunteers were not statistically different [15]. We also investigated the expression of GRAIL in the peripheral blood of *Il-10*$^{-/-}$ mice. *Il-10*$^{-/-}$ mice develop spontaneous chronic intestinal inflammation at approximately 4 weeks of age, while *Il-10*$^{+/-}$ mice lack this inflammation. The levels of GRAIL mRNA in the peripheral blood CD4$^+$ T cells were lower in *Il-10*$^{-/-}$ mice

Table 1 Clinical characteristics of the patients

	CD (active)	CD (remission)	HV
Gender (M/F)	13/4	14/6	16/6
Disease type (ileal/colonic/ileo-colonic)	9/2/6	8/7/5	–
Age[a]	38.2 (±11.7)	40.0 (±13.9)	35.6 (±7.7)
CDAI[a]	251.1 (±69.3)**	85.2 (±30.9)	–
CRP (mg/l)[a]	13.6 (±20.3)***	4.1 (±6.6)	–

CD Crohn's disease, *HV* healthy volunteers, *CDAI* Crohn's disease activity index, *CRP* C-reactive protein

[a] Mean (±SEM)

** $P < 0.05$

*** $P < 0.01$, compared with CD in remission

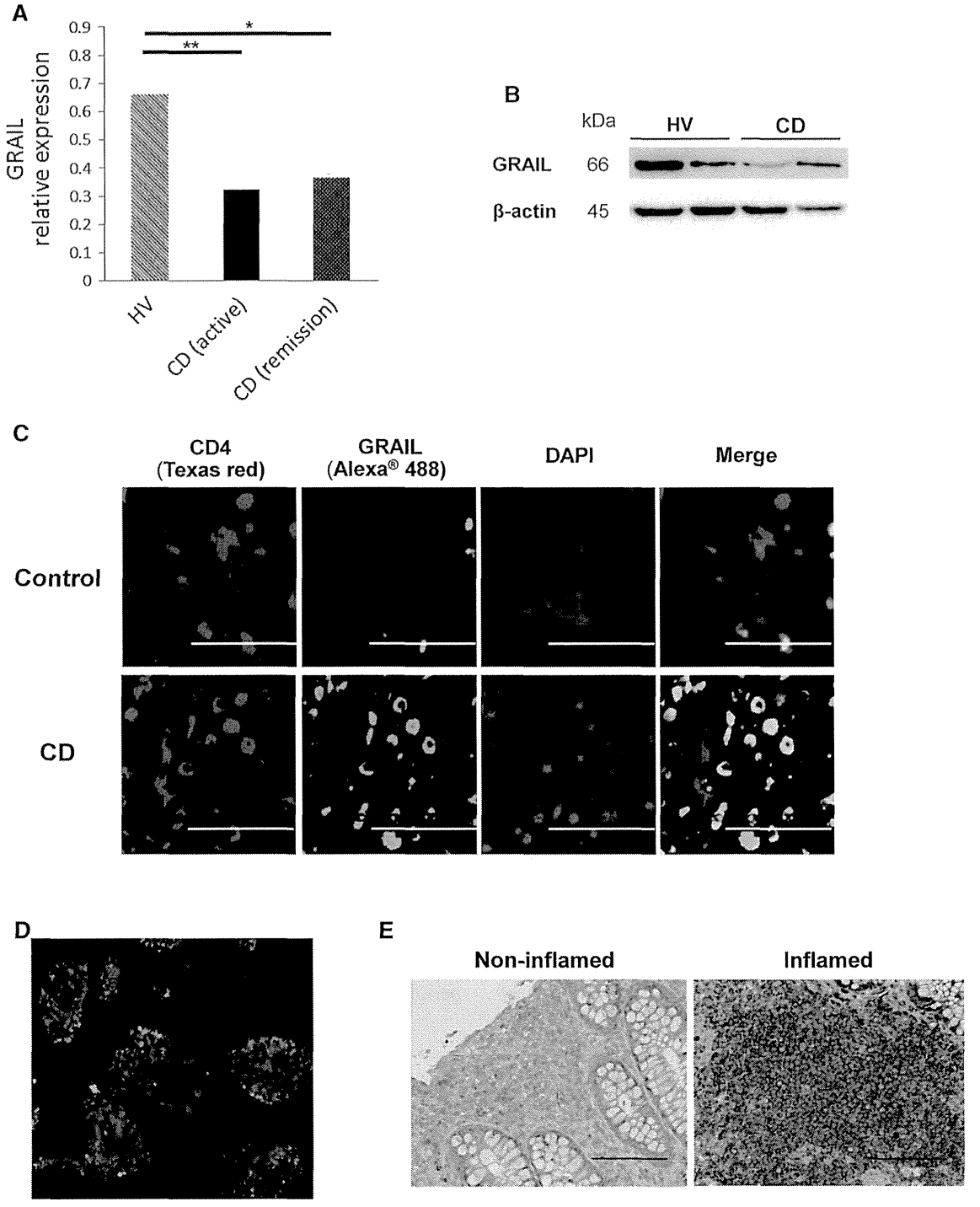

Fig. 1 Expression of GRAIL is lower in the peripheral $CD4^+$ T cells, but higher in the LP of Crohn's disease. **a** Levels of GRAIL mRNA in the peripheral blood $CD4^+$ T cells isolated from 22 healthy volunteers (HV), 17 active patients with CD, and 20 patients with CD in remission were analyzed by qRT-PCR. GRAIL mRNA was significantly lower in the $CD4^+$ T cells of active and remissive CD patients than in HV. Data are shown as the mean + SEM. $^*P < 0.05$; $^{**}P < 0.01$. **b** GRAIL protein expressions of the peripheral blood $CD4^+$ T cells isolated from HV and the patients of CD were analyzed by western blot. Representative blots from HV and active CD patients are shown. **c** Immunohistochemical staining of non-inflamed colonic mucosa of colon cancer patients (control) and inflamed mucosa of CD patients. Colonic tissues were stained with Texas red-labeled anti-CD4 antibody (*red*), Alexa®488-labeled anti-GRAIL antibody (*green*) and DAPI (*blue*). *Scale bar* 50 μm. **d** The LP mononuclear cells of patients with CD were stained with CD4-Texas red (*red*), GRAIL-Alexa®488 (*green*) and DAPI (*blue*), and subjected to three-dimensional, structured illumination microscopy. **e** Specimens from non-inflamed and inflamed colonic mucosa were stained by anti-GRAIL antibody and color was developed by 3, 3′-diaminobenzidine. GRAIL-stained cells (*brown*) were massively observed in the LP of inflamed lesion. *Scale bar* 100 μm (color figure online)

than in *Il-10*$^{+/-}$ mice, consistent with the reduced protein expression of GRAIL in *Il-10*$^{-/-}$ mice (Supplementary Fig. 1A, B).

Expressions of GRAIL mRNA and protein in CD4$^+$ T cells in the intestinal lamina propria of the patients with CD

Next, we examined GRAIL protein expression in the intestinal LP of CD patients and control subjects by immunohistochemistry. The GRAIL-positive cells were highly observed in the intestinal CD4$^+$ T cells of the patients with CD in contrast to the infrequent GRAIL-positive cells in control subjects (Fig. 1c, Supplementary Fig. 2A). When we investigated the GRAIL protein expression by three-dimensional, structured illumination microscopy, GRAIL was ubiquitously expressed in the cytoplasm, suggestive of endosomes, of CD4$^+$ T cells in the intestinal LP of the patients with CD (Fig. 1d). In addition, the expression of GRAIL was massively observed in severely inflamed lesions with erosions and ulcers, rather than in non-inflamed lesions (Fig. 1e). In contrast, GRAIL mRNA expressions were not significantly different between the colonic tissue samples of CD and the control subjects (Supplementary Fig. 2B). Thus, we observed discrepant patterns of GRAIL expression between the peripheral blood and the intestinal LP in patients with CD.

Discrepant GRAIL mRNA and protein expressions in the colonic CD4$^+$ T cells of dextran sodium sulfate-induced colitis

We next investigated the expression of GRAIL in the lymphoid organs of DSS-induced, acute murine colitis. The level of GRAIL mRNA was significantly lower in the LP CD4$^+$ T cells of mice with DSS colitis than in those of mice untreated with DSS (Fig. 2a). In contrast, the GRAIL protein level in the LP CD4$^+$ T cells of colitic mice was significantly higher than that of untreated mice (Fig. 2b, c). The level of GRAIL mRNA in CD4$^+$ T cells was significantly higher in the LP than in the SP or MLN of untreated mice (Fig. 2a). In contrast, the GRAIL protein level was lower in the CD4$^+$ T cells in the LP than in those in the SP and MLN of untreated mice (Fig. 2b, c).

Discrepant expressions between the GRAIL mRNA and protein in the colonic CD4$^+$ T cells of *Il-10*$^{-/-}$ mice

When the GRAIL mRNA was compared between *Il-10*$^{-/-}$ and *Il-10*$^{+/-}$ mice, the level of GRAIL mRNA was significantly lower in the LP CD4$^+$ T cells of colitic *Il-10*$^{-/-}$ mice than those of non-colitic *Il-10*$^{+/-}$ mice (Fig. 2d). In contrast, the level of GRAIL protein in the LP was significantly higher in *Il-10*$^{-/-}$ mice than in *Il-10*$^{+/-}$ mice (Fig. 2e, f). Thus, the GRAIL mRNA and protein expression patterns of *Il-10*$^{-/-}$ mice were similar to those of mice with DSS colitis in terms of the discrepancy of GRAIL mRNA and protein levels in the LP between colitic and non-colitic mice. In addition, there was a discrepancy of GRAIL mRNA and protein between the SP and LP in the non-colitic mice, showing high mRNA levels but low protein levels of GRAIL in the LP compared to the SP. It has been shown that the specific deubiquitinating enzyme (DUB), Otub-1, induces degradation of GRAIL [10, 25]. We speculated that the suppression of Otub-1 might upregulate GRAIL expression via the abrogation of deubiquitination of GRAIL in the LP of colitic mice. The levels of Otub-1 mRNA, however, were not significantly different among CD4$^+$ T cells in the SP, MLN, and LP of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (Supplementary Fig. 3A). In addition, the levels of ubiquitination of the GRAIL protein in the CD4$^+$ T cells of SP and LP were not different in both *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (Supplementary Fig. 3B). These results indicated that Otub-1 does not play a major role for the regulation of GRAIL in murine colitis.

miR-290-5p as a novel miRNA related to the regulation of GRAIL

miRNAs, a novel class of non-coding small RNAs, repress gene expression by binding to the 3′UTR of target messenger RNAs, thereby suppressing protein expression [26]. We screened miRNAs that were highly expressed in the LP relative to the SP by miRNA microarray, using samples of SP and LP CD4$^+$ T cells isolated from *Il-10*$^{+/-}$ mice, because there was a discrepancy between the mRNA and protein levels in these two lymphoid organs. The miRNA microarray revealed upregulation of 58 miRNAs in the LP, compared with SP (GEO accession number; GSE40891). Among these upregulated miRNAs, computational predictions revealed that miR-26b, miR-295, miR-330, miR-290-5p, and miR-290-3p had a potential to bind to the 3′UTR of GRAIL and miR-290-5p was expressed more than two-fold higher in the LP than in the SP (Table 2). The seed lesion of miR-290-5p completely coincided with the putative GRAIL-3′UTR (Fig. 3A). We next analyzed the expression of miR-290-5p in the CD4$^+$ T cells of the systemic and mucosal lymphoid tissues of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice by qRT-PCR. miR-290-5p was confirmed to be expressed significantly higher in the LP than in the SP of *Il-10*$^{+/-}$ mice (Fig. 3b). In addition, the expression of miR-290-5p in the CD4$^+$ T cells of the LP was significantly higher in *Il-10*$^{+/-}$ mice than in *Il-10*$^{-/-}$ mice. The miR-290-5p levels in the SP and MLN were not significantly different between *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (Fig. 3b).

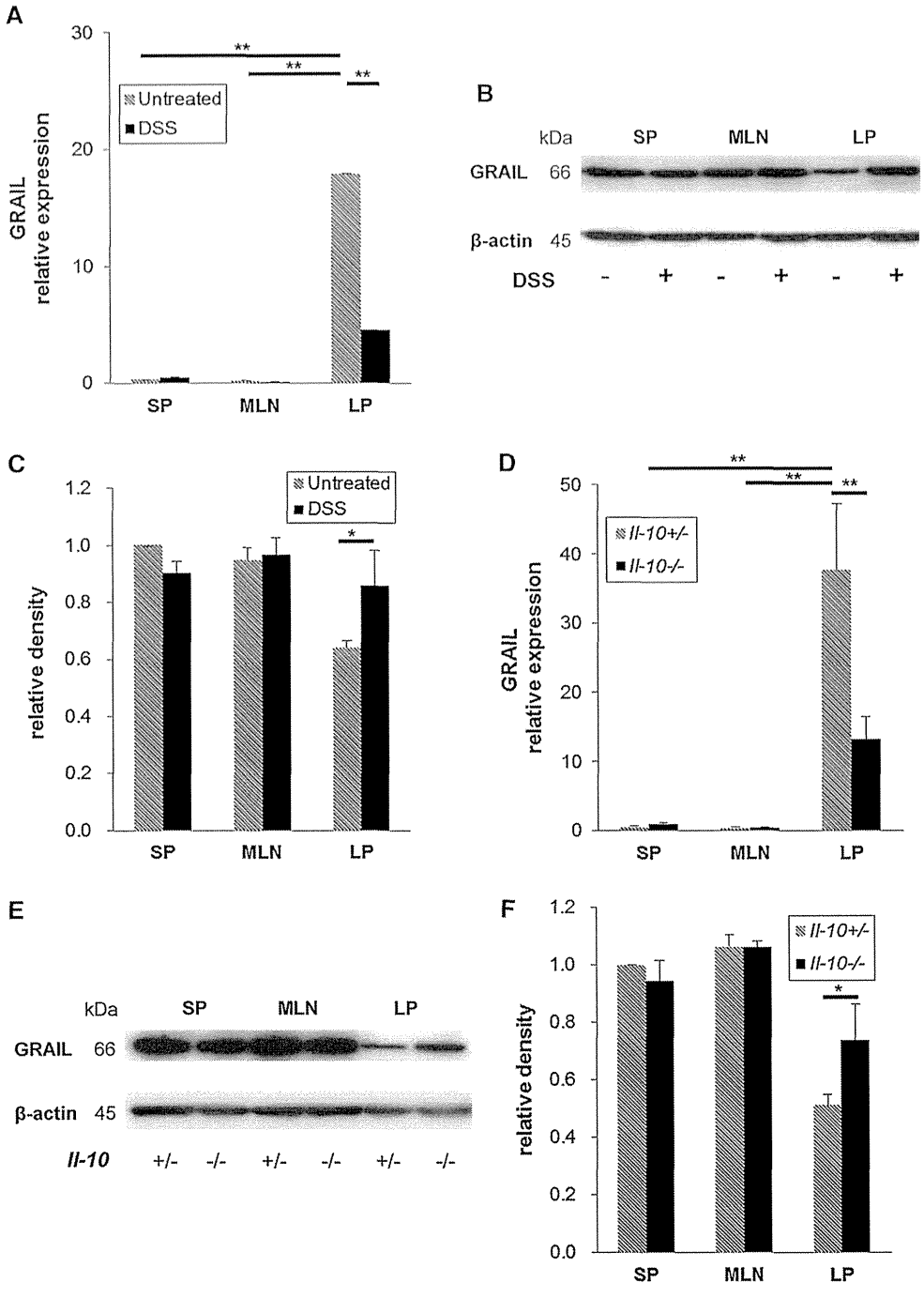

Fig. 2 Discrepancy between GRAIL mRNA and protein expressions in the lamina propria of murine colitis. **a** The levels of GRAIL mRNA were evaluated in mice induced with DSS colitis. GRAIL mRNA was significantly higher in the $CD4^+$ T cells of the LP than in those of the SP and MLN in untreated mice. GRAIL mRNA was significantly lower in the LP of DSS-treated mice (*solid bar*) than mice untreated with DSS (*shaded bar*; $n = 8$ in each group; $^{**}P < 0.01$). **b** The GRAIL protein levels in the $CD4^+$ T cells isolated from lymphoid tissues were analyzed by western blot in mice treated with DSS (+) or without DSS (−). Representative pictures of three independent experiments are shown. **c** Band intensities were quantified by densitometry. The data were expressed as mean + SEM from three separate experiments. $^{*}P < 0.05$. **d** The mRNA expressions of GRAIL in $CD4^+$ T cells of the SP, MLN, and LP of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (age 10–12 weeks) were determined by qRT-PCR ($n = 8$). The levels of GRAIL mRNA in the LP were significantly higher than in other lymphoid tissues in *Il-10*$^{+/-}$ mice. GRAIL mRNA of the LP was higher in *Il-10*$^{+/-}$ mice (*shaded bar*) than in *Il-10*$^{-/-}$ mice (*solid bar*; $^{**}P < 0.01$). E. The levels of GRAIL protein in $CD4^+$ T cells of the SP, MLN, and LP of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice were analyzed by western blot. Representative pictures from three independent experiments are shown. **f** Band intensities were quantified by densitometry. The data were expressed as mean + SEM from three separate experiments. $^{*}P < 0.05$

Table 2 miRNAs upregulated in the lamina propria and predicted to bind to GRAIL

miRNA	Ratio (LP/SP)
mmu-miR-290-5p	2.15
mmu-miR-295	1.26
mmu-miR-330	1.18
mmu-miR-290-3p	1.16
mmu-miR-26b	1.15

We introduced DO11.10 cells with an anti-miR-290 inhibitor to confirm that miR-290-5p inhibits GRAIL protein expression. The expression of GRAIL protein was increased after the introduction of an anti-miR-290 inhibitor (Fig. 3c). Next, to confirm that miR-290-5p can inhibit the translation of GRAIL mRNA, we generated a plasmid vector, pMIR-GRAIL-3′UTR, in which the putative miR-290-5p binding site of GRAIL was inserted downstream of the firefly luciferase open-reading frame, and conducted a luciferase reporter assay using a pMIR-GRAIL-3′UTR vector co-transfected with the miR-290 precursor. Firefly luciferase activity was significantly decreased by transfection of the miR-290 precursor compared to the negative control (Fig. 3d). These results confirmed that miR-290-5p can inhibit expression of GRAIL protein via binding to the GRAIL-3′UTR.

T cells overexpressing GRAIL express regulatory markers

The plasmid vector designed to express pAcGFP1 and GRAIL was transfected in DO11.10 cells and we evaluated the expression of markers for regulatory T cells, e.g., FoxP3, GITR, and CTLA-4 [27]. The *grail* mRNA was confirmed to be expressed in the cells transfected with pAcGFP1-GRAIL (Fig. 4a). The *foxp3* and *gitr* mRNA levels were significantly higher in the cells transfected with pAcGFP1-GRAIL than in those transfected with the control vector (Fig. 4a). The protein levels of IL-10 and TGF-β were significantly lower in the cells with transfection of pAcGFP-GRAIL than in the cells transfected with the control GFP vector (Fig. 4b). These results are consistent

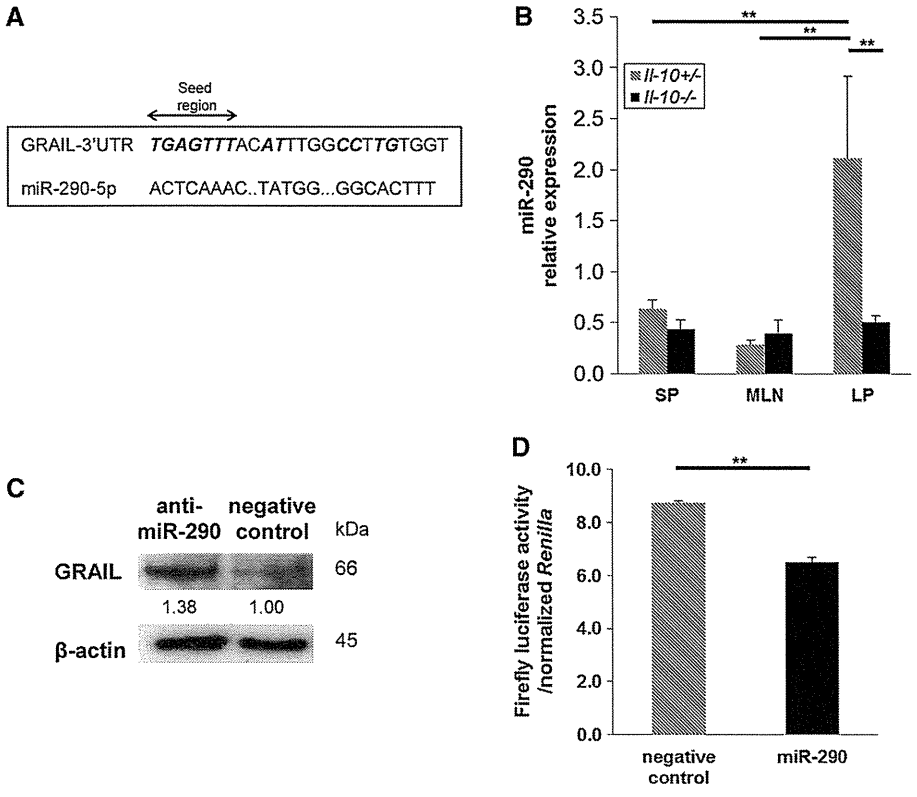

Fig. 3 miR-290-5p regulates GRAIL expression in vitro. **a** The sequences of miR-290-5p and 3′UTR of GRAIL. **b** Expression of miR-290-5p was determined by qRT-PCR. Expression of miR-290-5p was significantly higher in the LP of *Il-10*$^{+/-}$ mice than in the other lymphoid tissues and was significantly lower in the LP of *Il-10*$^{-/-}$ mice than of *Il-10*$^{+/-}$ mice. The levels of miR-290-5p normalized by snoRNA135 are shown ($n = 8$, $^{**}P < 0.01$). **c** Expression of GRAIL protein was determined by western blotting after the transfection of DO11.10 cells with an anti-miR-290-5p inhibitor. Relative protein levels were determined by densitometry. **d** Luciferase activity was determined by the transfection of NIH-3T3 with GRAIL-3′UTR reporter together with miR-290 precursor. Luciferase activity was significantly decreased by miR-290 precursor compared with the pre-miR^{TM} miRNA negative control ($n = 6$, $^{**}P < 0.01$)

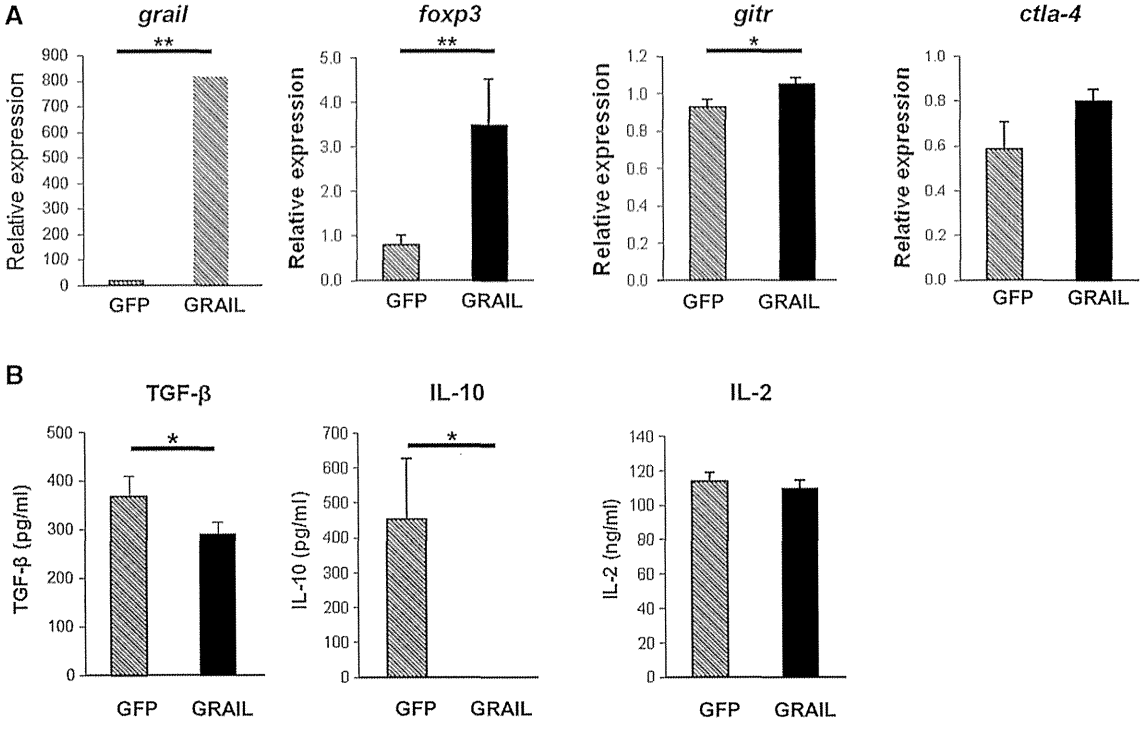

Fig. 4 Forced expression of GRAIL converts DO11.10 cells to a regulatory phenotype. **a** Either pAcGFP-GRAIL vector or empty pAcGFP vector was transfected to DO11.10 cells, and *grail*, *foxp3*, *gitr*, and *ctla-4* mRNA expressions in AcGFP positive cells were determined by qRT-PCR. Expressions of *foxp3* and *gitr* mRNA were significantly higher in GRAIL-overexpressing cells than in cells transfected with control vector ($n = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$). **b** DO11.10 cells transfected with pAcGFP1-GRAIL or empty vector were cultured in vitro with anti-CD3 and anti-CD28 mAb for 24 h and the culture supernatants were analyzed for TGF-β, IL-10, and IL-2 by ELISA. The expressions of TGF-β and IL-10 were significantly higher in the control group than in the GRAIL group ($n = 5$; $^{*}P < 0.05$)

with the previous report showing forced expression of GRAIL converts DO11.10 to a suppressor phenotype, irrespective of the suppressive cytokine expressions [12]. The suppressive effect on proliferative activity of the naïve responder cells by the GRAIL-expressing DO11.10 T cell line has also been demonstrated [12]. Similarly, cell division was decreased in the presence of GRAIL-transfected cells when compared to the GFP control cells (Supplementary Fig. 4). Thus, GRAIL expression converted T cells to a regulatory phenotype independent of regulatory cytokines, such as IL-10 and TGF-β.

Effect of GRAIL^{high} T cell-transfer on intestinal inflammation in murine colitis induced by dextran sodium sulfate

To assess the function of GRAIL, C57BL/6J mice orally administered 2 % DSS were injected with GRAIL-overexpressing DO11.10 (GRAIL^{high}) T cells. The injection of GRAIL^{high} T cells significantly suppressed the body-weight loss of mice with DSS colitis (Fig. 5a). The length of the colon was significantly longer in mice transferred with GRAIL^{high} T cells than in the control group (Fig. 5b, c). The cellular profile was not different between recipient mice transferred with GRAIL^{high} cells and the control mice (Supplementary Fig. 5). The histology of the colon section showed less infiltration of inflammatory cells in mice transferred with the GRAIL^{high} cells when compared with the control group, and myeloperoxidase activity tended to be lower in the GRAIL^{high} group than the control group, but these results did not reach statistical significance (Fig. 5d, e, Supplementary Fig. 6A). The GRAIL^{high} T cells were detected mainly in the abdomen by in vivo imaging system (Supplementary Fig. 6B) and GFP-positive cells were confirmed to be located in the colonic LP of mice transferred with GRAIL^{high} T cells (Fig. 5f).

GRAIL-positive cells express FoxP3 in the lamina propria of CD patients

We further examined the expression of FoxP3 and GRAIL in the colonic lamina propria of CD patients and control subjects. Many GRAIL-positive cells were co-stained with anti-FoxP3 antibody, especially in the inflamed, colonic LP of the patients with CD (Fig. 6).

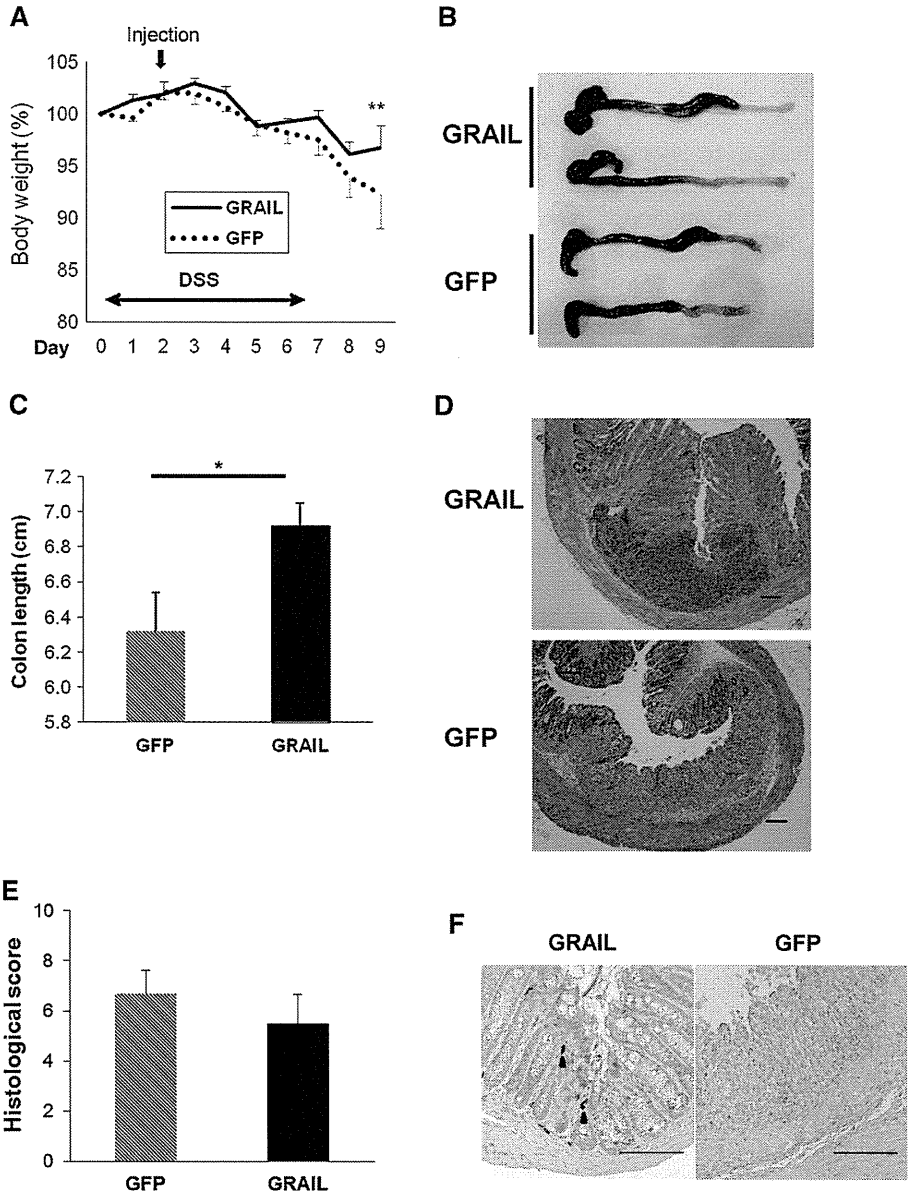

Fig. 5 Transfer of GRAIL^{high} T cells suppresses colonic inflammation induced by dextran sodium sulfate. **a** Mice induced with DSS colitis were injected with either GRAIL^{high} DO11.10 (GRAIL) or the cells transfected with the GFP-expressing empty vector (GFP) on day 2. Mice injected with GRAIL^{high} cells were significantly protected from body-weight loss compared to the control group ($n = 6$; $^{**}P < 0.01$). **b** Macroscopic pictures of the colon with or without GRAIL^{high} cell-transfer. **c** The colon lengths of mice with GRAIL^{high} cell-transfer were significantly longer than those of the control group ($n = 6$; $^{*}P < 0.05$). **d** Representative H&E sections of the distal colon of mice injected with either GRAIL^{high} or control cells (magnification, ×10). *Bar* 100 μm. **e** Histological scores of GRAIL^{high} and control groups ($n = 6$, not significant). **f** Colonic sections of mice with DSS colitis, injected with GRAIL^{high} cells or control cells, were stained with an anti-GFP antibody. The GFP-positive cells expressing GRAIL (*arrow*) were observed in the colonic lamina propria (*scale bar* 100 μm)

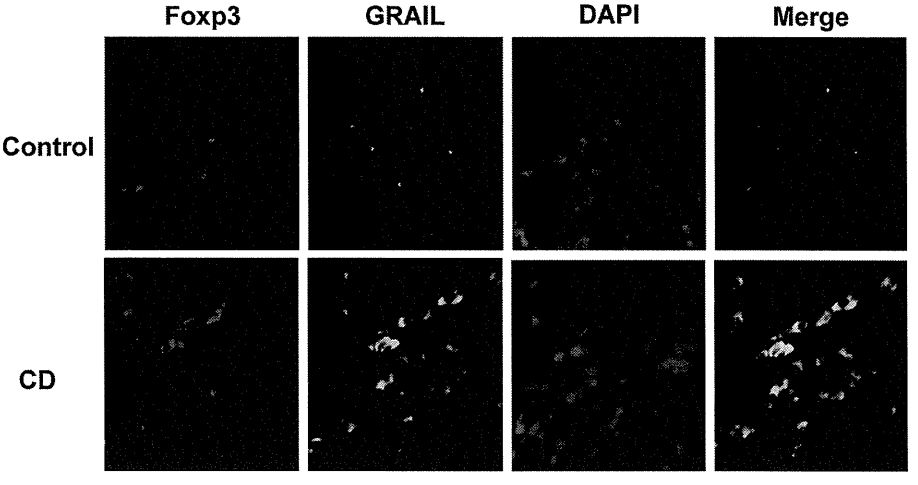

Fig. 6 Immunohistochemical staining of FoxP3 and GRAIL in the non-inflamed, colonic mucosa of colon cancer patients (control) and in the inflamed mucosa of CD patients. Colonic tissues were stained with anti-GRAIL antibody followed by Alexa®488-labeled anti-rabbit IgG (*green*), mouse anti-FoxP3 antibody followed by Texas red-labeled anti-mouse IgG (*red*), and DAPI (*blue*). The representative pictures are shown (color figure online)

Discussion

Our present study demonstrated for the first time the expression of GRAIL in the peripheral blood of CD patients and mice with experimental colitis. We also demonstrated the unique local regulation of GRAIL by miRNA. We have previously reported that GRAIL mRNA expression was increased in the peripheral blood $CD4^+$ T cells of patients with UC in the remissive state, while the levels of GRAIL were not different between HV and UC in the active state [15]. In the present study, we demonstrated that GRAIL mRNA and protein expressions were lower in the peripheral blood $CD4^+$ T cells of the patients with CD than in those of healthy subjects, irrespective of the activity of the patients with CD. Thus, the expression pattern of GRAIL in the peripheral blood is completely different between UC and CD. Our present results suggest that systemic T-cell anergy is abrogated in patients with CD, even in patients who have established clinical remission. Decrease of GRAIL expression in the peripheral blood of $CD4^+$ T cells may be used as a new biomarker of CD. Our present results showing difference in the expression of GRAIL between CD and UC are consistent with the fact that T-cell cycle abnormalities are present in CD, indicating a state of hyperreactivity compatible with a loss of tolerance in contrast to a hyporeactive state in UC [28]. The precise mechanisms of the difference of T cell states are unknown, and the differences in anergic immune status between CD and UC need to be further investigated.

Our study demonstrated the unique expression and regulation of GRAIL in the LP in physiological and inflammatory conditions. In non-colitic mice, GRAIL protein expression was lower in LP $CD4^+$ T cells, regardless of the high expression of GRAIL mRNA, in comparison to the SP $CD4^+$ T cells. In terms of the discrepancy between mRNA and protein levels of GRAIL in the LP, we initially speculated that GRAIL protein degradation by Otub-1 might mediate the discrepancy of GRAIL mRNA and protein expressions [25]. Otub-1 is a member of the deubiquitinating enzymes group (DUBs) with the capacity to cleave proteins at the ubiquitin-protein bond using its cysteine protease domain. GRAIL expression is reduced by Otub-1 expression via the abrogation of ubiquitin-specific protease (USP) 8-mediated deubiquitination and stabilization of GRAIL [14, 29]. Otub-1 expression, however, was not found to be associated with the local regulation of GRAIL in this study. Recent studies have shown that miRNAs are associated with the pathogenesis of CD using plasma and colonic tissues [30], but the functions of these miRNAs are not yet clarified. In the present study, we firstly identified a novel system to suppress GRAIL protein expression by miR-290 in the normal intestinal mucosa. Elevation of miR-290 was observed in the LP $CD4^+$ T cells of control mice, but not in those of inflamed colon or in other lymphoid organs (SP, MLN). In the non-inflammatory condition, translation of GRAIL was suggested to be suppressed by miR-290. In contrast, the GRAIL protein expression might not be decreased because the expression of miR-290 was not increased in the inflammatory condition. Thus, GRAIL protein expression turned out to be higher in the LP $CD4^+$ cells from colitic mice than in those from control mice. In addition to the regulation by miRNA, the $GRAIL^{high}$ T cells migrated to the inflamed intestine (Fig. 5f, Supplementary Fig. 6B). The alteration of cellular migration after the expression of GRAIL must be further investigated.

The regulatory role of $GRAIL^{high}$ DO11.10 cells has already been demonstrated in vitro [12]. We confirmed a regulatory function of $GRAIL^{high}$ T cells by investigating the proliferative activity of fluorescent-labeled T cells (Supplementary Figure 4). $GRAIL^{high}$ T cells did not exhibit either the upregulation of regulatory cytokines or the decrease of the effector cytokines (Fig. 4b). These results were consistent with the results showing that $GRAIL^{high}$ T cells exhibit a regulatory function without the induction of regulatory cytokines [12]. GRAIL-positive T cells were also shown to highly express FoxP3. In addition, we demonstrated that transfer of $GRAIL^{high}$ T cells partially reduced intestinal inflammation in murine colitis. These results indicated that GRAIL-expressing T cells have a potential to prevent acceleration of inflammation in the intestinal mucosa. Although cells co-expressing FoxP3 and GRAIL were highly detected in the inflamed mucosa of the colon of CD patients, intestinal inflammation was evident in the CD patients. An ameliorative effect of GRAIL may not be so powerful as to establish a complete reduction of inflammation. We may have to investigate whether the $GRAIL^{high}$ cells still maintain a regulatory function in the local inflammatory site. The pathophysiological roles of GRAIL-expressing T cells are yet to be further investigated in relation to luminal antigens and commensal bacteria that reside in the intestine.

It should be noted as a limitation of our study that no identical miRNA for murine miR-290 was found in the database for humans (miRBase). Because the GRAIL expression pattern in the LP $CD4^+$ T cells was similar in patients with CD and *Il-10*$^{-/-}$ mice, it was speculated that the GRAIL of CD patients is regulated by a similar mechanism to that of *Il-10*$^{-/-}$ mice. This regulatory mechanism of GRAIL by miRNA can be novel for controlling immune status via the alteration of the anergic status of $CD4^+$ T cells, and more investigation is required to search for the miRNAs that control GRAIL in humans.

In conclusion, our study revealed a reduction of GRAIL expression in the peripheral blood of patients with CD. We also showed the presence of GRAIL-expressing $CD4^+$

T cells in the inflamed intestinal mucosa and a novel regulatory mechanism of GRAIL by miRNA. Our understanding of miRNA biogenesis will provide insight into the cellular functions in the inflammatory processes of IBD. A more precise investigation of the regulatory mechanisms of GRAIL would help to explore powerful therapeutic strategies for controlling inflammation by inducing anergy.

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Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Leone V, Chang EB, Devkota S. Diet, microbes, and host genetics: the perfect storm in inflammatory bowel diseases. J Gastroenterol. 2013;48:315–21.
2. Hamilton MJ, Snapper SB, Blumberg RS. Update on biologic pathways in inflammatory bowel disease and their therapeutic relevance. J Gastroenterol. 2012;47:1–8.
3. Arimura Y, Isshiki H, Onodera K, Nagaishi K, Yamashita K, Sonoda T, et al. Characteristics of Japanese inflammatory bowel disease susceptibility loci. J Gastroenterol. 2013. doi:10.1007/s00535-013-0866-2.
4. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol. 2006;3:390–407.
5. Anandasabapathy N, Ford GS, Bloom D, Holness C, Paragas V, Seroogy C, et al. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. Immunity. 2003;18:535–47.
6. Iweala OI, Nagler CR. Immune privilege in the gut: the establishment and maintenance of non-responsiveness to dietary antigens and commensal flora. Immunol Rev. 2006;213:82–100.
7. Kraus TA, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. Gastroenterology. 2004;126:1771–8.
8. Mueller DL. E3 ubiquitin ligases as T cell anergy factors. Nat Immunol. 2004;5:883–90.
9. Lineberry NB, Su LL, Lin JT, Coffey GP, Seroogy CM, Fathman CG. Cutting edge: the transmembrane E3 ligase GRAIL ubiquitinates the costimulatory molecule CD40 ligand during the induction of T cell anergy. J Immunol. 2008;181:1622–6.
10. Su LL, Iwai H, Lin JT, Fathman CG. The transmembrane E3 ligase GRAIL ubiquitinates and degrades CD83 on CD4 T cells. J Immunol. 2009;183:438–44.
11. Nurieva RI, Zheng S, Jin W, Chung Y, Zhang Y, Martinez GJ, et al. The E3 ubiquitin ligase GRAIL regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation. Immunity. 2010;32:670–80.
12. MacKenzie DA, Schartner J, Lin J, Timmel A, Jennens-Clough M, Fathman CG, et al. GRAIL is up-regulated in CD4+ CD25+ T regulatory cells and is sufficient for conversion of T cells to a regulatory phenotype. J Biol Chem. 2007;282:9696–702.
13. Schartner JM, Singh AM, Dahlberg PE, Nettenstrom L, Seroogy CM. Recurrent superantigen exposure in vivo leads to highly suppressive CD4+ CD25+ and CD4+ CD25$^-$ T cells with anergic and suppressive genetic signatures. Clin Exp Immunol. 2009;155:348–56.
14. Whiting CC, Su LL, Lin JT, Fathman CG. GRAIL: a unique mediator of CD4 T-lymphocyte unresponsiveness. FEBS J. 2011;278:47–58.
15. Egawa S, Iijima H, Shinzaki S, Nakajima S, Wang J, Kondo J, et al. Upregulation of GRAIL is associated with remission of ulcerative colitis. Am J Physiol Gastrointest Liver Physiol. 2008;295:G163–9.
16. MacDonald TT, Monteleone I, Fantini MC, Monteleone G. Regulation of homeostasis and inflammation in the intestine. Gastroenterology. 2011;140:1768–75.
17. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl. 1989;170:2–6; discussion 16–9.
18. Podolsky DK. Inflammatory bowel disease (1). N Engl J Med. 1991;325:928–37.
19. Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. Gastroenterology. 1976;70:439–44.
20. Fujihashi K, Taguchi T, Aicher WK, McGhee JR, Bluestone JA, Eldridge JH, et al. Immunoregulatory functions for murine intraepithelial lymphocytes: gamma/delta T cell receptor-positive (TCR+) T cells abrogate oral tolerance, while alpha/beta TCR+ T cells provide B cell help. J Exp Med. 1992;175:695–707.
21. Takahashi I, Kiyono H, Hamada S. CD4+ T-cell population mediates development of inflammatory bowel disease in T-cell receptor alpha chain-deficient mice. Gastroenterology. 1997;112:1876–86.
22. Sudo H, Mizoguchi A, Kawauchi J, Akiyama H, Takizawa S. Use of non-amplified RNA samples for microarray analysis of gene expression. PLoS ONE. 2012;7:e31397.
23. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet. 2001;29:365–71.
24. Dohi T, Ejima C, Kato R, Kawamura YI, Kawashima R, Mizutani N, et al. Therapeutic potential of follistatin for colonic inflammation in mice. Gastroenterology. 2005;128:411–23.
25. Soares L, Seroogy C, Skrenta H, Anandasabapathy N, Lovelace P, Chung CD, et al. Two isoforms of otubain 1 regulate T cell anergy via GRAIL. Nat Immunol. 2004;5:45–54.
26. Sinkkonen L, Hugenschmidt T, Berninger P, Gaidatzis D, Mohn F, Artus-Revel CG, et al. MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol. 2008;15:259–67.
27. Corthay A. How do regulatory T cells work? Scand J Immunol. 2009;70:326–36.
28. Sturm A, Leite AZ, Danese S, Krivacic KA, West GA, Mohr S, et al. Divergent cell cycle kinetics underlie the distinct functional capacity of mucosal T cells in Crohn's disease and ulcerative colitis. Gut. 2004;53:1624–31.
29. Lin JT, Lineberry NB, Kattah MG, Su LL, Utz PJ, Fathman CG, et al. Naive CD4 t cell proliferation is controlled by mammalian target of rapamycin regulation of GRAIL expression. J Immunol. 2009;182:5919–28.
30. Dalal SR, Kwon JH. The role of MicroRNA in inflammatory bowel disease. Gastroenterol Hepatol (N Y). 2010;6:714–22.

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Amelioration of Small Bowel Injury by Switching from Nonselective Nonsteroidal Anti-Inflammatory Drugs to Celecoxib in Rheumatoid Arthritis Patients: A Pilot Study

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Key Words
Nonsteroidal anti-inflammatory drugs · Cyclooxygenase-2 selective inhibitor · Small bowel injury · Video capsule endoscopy

Abstract
Background/Aims: Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in patients with rheumatoid arthritis (RA) but have several side effects including mucosal damage in the small intestine. We aimed to evaluate whether the small bowel injury is ameliorated by switching from nonselective NSAIDs to celecoxib in patients with RA. ***Methods:*** Sixteen patients with RA who were treated with nonselective NSAIDs were enrolled in this study. Nonselective NSAIDs were converted to celecoxib for 12 weeks. Capsule endoscopy was performed before and after treatment with celecoxib. Videos were screened by gastroenterologists blinded to the patients' treatment. ***Results:*** Before the administration of celecoxib, reddened folds, denuded areas, petechiae/red spots and mucosal breaks were observed in 63, 63, 88 and 69% of the patients, respectively. In the 14 patients who completed this study, conversion to celecoxib significantly reduced the number of petechiae/red spots, the number of mucosal breaks, and Lewis scores. RA activity and cytokine levels in the peripheral blood were not significantly different before and after treatment with celecoxib. ***Conclusions:*** The incidence of small bowel injury by nonselective NSAIDs is high in patients with RA. Conversion from nonselective NSAIDs to celecoxib can be useful for protecting patients with RA from small bowel injury.

Introduction

Recent progress in the development of biologics, including disease-modifying antirheumatic drugs, has changed the treatment strategy for rheumatoid arthritis (RA) [1]. However, nonsteroidal anti-inflammatory drugs (NSAIDs) are still widely used because of their high efficacy for pain control and cost-effectiveness [2]. In spite of the usefulness of NSAIDs for reducing pain, patients who take NSAIDs are at a high risk for severe in-

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jury in the mucosa of the stomach and duodenum [3, 4]. Chronic NSAID users are estimated to suffer from ulcer complications (bleeding or perforation) at a rate of 1–4% each year, and NSAID use has been shown to be associated with up to 2,500 deaths per year in the UK population [5]. Previous randomized trials primarily focused on damage in the upper gastrointestinal (GI) tract, but recent reports have shown that lower GI events were also observed in patients with RA who use NSAIDs [6, 7].

NSAIDs act by inhibiting cyclooxygenase (COX), which converts arachidonic acid to prostaglandins [8, 9]. COX exists in two isoforms: COX-1, an essential enzyme to produce prostaglandins involved in the cytoprotective functions in the GI mucosa [10, 11]; COX-2, a predominantly cytokine-induced enzyme, produces prostaglandins to mediate pain and inflammation. Nonselective NSAIDs inhibit both COX-1 and COX-2, and gastroduodenal injury is suggested to mainly result from COX-1 inhibition. A selective COX-2 inhibitor has a characteristic of reducing mucosal damage compared to NSAIDs [12, 13], and celecoxib is one of the most commonly used selective COX-2 inhibitors. Several randomized controlled trials have compared the efficacy and side effects between celecoxib and nonselective NSAIDs and have shown that celecoxib reduced the relative risk of gastroduodenal ulcers compared to nonselective NSAIDs to 79% [12, 14, 15]. Over 24 weeks, the prevalence of endoscopically identified gastroduodenal ulcers in patients with RA taking celecoxib was nearly 4-fold lower than that of diclofenac. In addition, the incidence of mucosal injury to the small intestine was significantly lower in healthy subjects who received celecoxib than the subjects who received nonselective NSAIDs plus omeprazole [16, 17]. Available data, however, were limited to studies regarding the administration of nonselective NSAIDs or celecoxib in healthy volunteers.

Recently, video capsule endoscopy (VCE) enabled noninvasive visualization of the whole small intestine [18, 19]. VCE revealed approximately 90% of patients with RA suffer from small bowel injury regardless of NSAID use [20]. To date, clinical trials investigating the effect of COX-2 selective inhibitors on damage to the small intestine are lacking. In addition, the efficacy of switching from nonselective NSAIDs to celecoxib has not been investigated in patients taking NSAIDs. In this study, patients with RA with long-term use of nonselective NSAIDs were evaluated for mucosal damage of the small intestine by VCE. After the evaluation of mucosal injuries in the small intestine, nonselective NSAIDs were switched to celecoxib. We investigated whether the switching from nonselective NSAIDs to celecoxib ameliorates small bowel injury.

Patients and Methods

Study Subjects

Patients with RA who were older than 20 years and had been regularly treated with NSAIDs (loxoprofen, diclofenac, indomethacin, etc.) for more than 3 months (median 24 months; range 4–164 months) were consecutively recruited in the Department of Respiratory Medicine, Allergy and Rheumatic Disease, Osaka University Hospital, from January 2009 to April 2011. Exclusion criteria included: patients under treatment with biologics [anti-tumor necrosis factor (TNF)-α antibody, anti-interleukin (IL)-6 receptor antibody, etc.], high-dose corticosteroids (>10 mg/day of prednisolone), aspirin, or anti-ulcer drugs (misoprostol, teprenone, rebamipide, etc.); active GI ulcers; known or suspected complete or partial stenosis of the small intestine; inflammatory bowel disease; severe cardiovascular disease; malignancies; mental disorders; severe liver, renal and hematopoietic diseases, and patients who were pregnant or breastfeeding. Participants were not allowed to change their medications during the study, except for NSAID and celecoxib usage.

Study Design

This was a prospective, open-label, endoscopist-blinded, single-arm, and single-center study. Patients with RA treated with NSAIDs were assigned to receive celecoxib 200 mg twice daily for 12 weeks after the discontinuation of NSAIDs. VCE was performed before and after the treatment with celecoxib. Patients were evaluated for changes in the serologic markers of RA, serum cytokine concentrations, joint pain, and adverse events or side effects of celecoxib. The study protocols and informed consent forms were approved by the institutional review boards of Osaka University Hospital, and all patients signed a written consent form before being included in the study. The trial is registered at UMIN-CTR, No. UMIN000002554. The full trial protocol can be accessed at http://www.umin.ac.jp/ctr/.

Video Capsule Endoscopy

The VCE (Given video capsule system with the PillCam SB1 capsule; Given Imaging Ltd., Yoqneam, Israel) was performed as previously described [21]. Briefly, one day before the VCE examination, the subjects were required to fast after 9 p.m. After the subjects were fitted with sensor array and data recorder on the following morning at 9 a.m., they swallowed the VCE. Patients were not allowed to drink fluids until 2 h after swallowing the VCE, and only a light meal was allowed after a subsequent 2 h. The subjects avoided exposure to magnetic fields or radio transmitters, which may have interfered with image capture; otherwise, they were allowed to perform their daily activities. The sensor array and data recorder were removed 8 h after swallowing the capsule and were returned to the investigator to download the images onto a computer workstation for analysis. Videos were blinded before analysis by deletion of information including patients' name, date of birth, and examination date.

Methodology for Reviewing the VCE

The blinded videos were viewed by a physician with vast experience in VCE, and thumbnail pictures of potential abnormalities were created. These thumbnails were reviewed by three investigators. The damage scale from a previous classification for acute NSAID-induced small bowel damage by Maiden et al. [21] was used

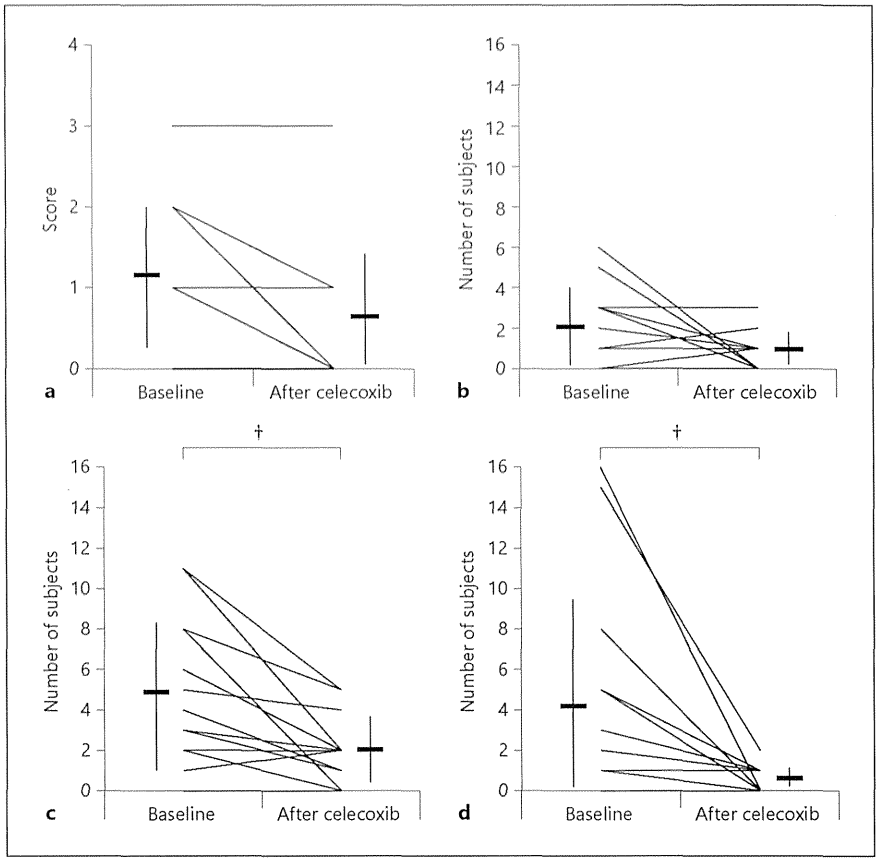

Fig. 1. Effect of the conversion from nonselective NSAIDs to celecoxib on the damage scale of small bowel lesions. The scores or number of lesions with each VCE finding [reddened folds (n = 14; **a**), denuded areas (n = 14; **b**), petechiae/red spots (n = 14; **c**) and mucosal breaks (n = 14; **d**)] were evaluated at baseline and after celecoxib treatment in the 14 patients who completed this study. † $p < 0.05$ after Bonferroni's correction. Bars indicate mean ± SD.

to evaluate mucosal injury as follows: category 1: reddened folds (≥1 valvulae conniventes showing discrete patchy or continuous erythema); category 2: denuded area (loss of villous architecture without a clear breach of the epithelium, may or may not be associated with surrounding erythema); category 3: petechiae/red spot (demarcated, usually circular, area of crimson mucosa with preservation of villi); category 4: mucosal break (mucosal erosions and/or ulcers, both represent discrete lesions with central pallor and surrounding hyperemia and loss of villi aphthae around the ulcer, and a punched out ulcer), graded by aphthae, circular ulcer, and punched out ulcer, and category 5: presence of blood. The severity of reddened folds was scored as 0 for none, 1 for mild, 2 for moderate, and 3 for severe. Additionally, the damage scale by Gralnek et al. [22], a capsule endoscopy scoring index for small bowel mucosal inflammatory change (Lewis score), was calculated for every patient by one endoscopist according to the judged thumbnails.

Assessing the Severity of RA

Changes in laboratory data including serum C-reactive protein (CRP), matrix metalloproteinase-3 (MMP-3) and blood hemoglobin (Hb) concentration were measured. Additionally, changes in serum cytokine concentrations were studied using the Bio-Plex human cytokine assay kit (Biorad, Hercules, Calif., USA) for platelet-derived growth factor-BB, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, interferon (IFN)-γ, IFN-inducible protein-10, monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated and normal T cell expressed and secreted, TNF-α, and vascular endothelial growth factor. Arthritis scores were evaluated before and after the treatment of celecoxib by Disease Activity Score in 28 joints calculated by using CRP (DAS28-CRP) as described previously [23]. The number of the joints with swelling (SJC28) and the number of the joints with tenderness (TJC28) among 28 joints were counted by the rheumatologist. The DAS28-CRP was calculated from SJC28, TJC28, pain visual analogue scale and serum CRP values.

Statistical Analysis

The mucosal injury scores and the blood test results were analyzed before and after the treatment with celecoxib among the 14 patients who had completed a previous study using Wilcoxon paired signed rank test and paired t test, respectively. The Bonferroni method was used to adjust for multiple comparisons; therefore, p values $<0.05/5 = 0.01$ for the mucosal injury parameters (fig. 1, 2) and $<0.05/4 = 0.0125$ for the blood test parameters (fig. 3) were considered statistically significant. JMP Pro version 10.0 (SAS Institute Inc., Cary, N.C., USA) was used for all the analyses.

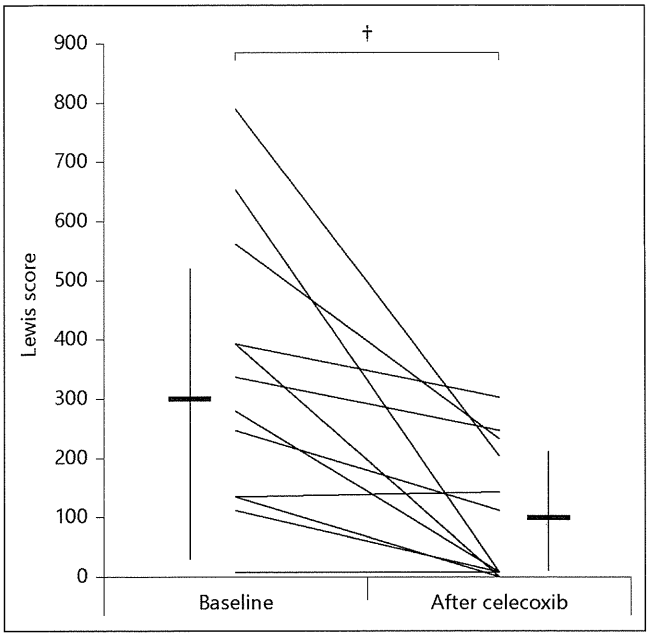

Fig. 2. Effect of conversion from nonselective NSAIDs to celecoxib on the scoring index for small bowel mucosal inflammatory change (Lewis score). The scoring index was evaluated at baseline and after celecoxib treatment in the 14 patients who completed this study. † $p < 0.05$ after Bonferroni's correction. Bars indicate mean ± SD.

Results

Patients

Sixteen patients underwent baseline VCE during the study period. Baseline characteristics for each subject are shown in table 1. Thirteen patients took loxoprofen; 3 patients took diclofenac, and 1 patient took indomethacin. One patient took both loxoprofen and diclofenac. Six patients took proton pump inhibitor (PPI) and 2 patients took histamine H2 receptor antagonist. Two patients dropped out because of general fatigue and disc herniation, which were not suggested as being related to celecoxib. Among the 14 patients who completed the study, no serious complications or side effects were observed.

Baseline VCE

Small bowel lesions were observed in 14 of 16 patients (88%) at baseline VCE. Reddened folds, denuded areas, petechiae/red spots and mucosal breaks were observed in 10 (63%), 10 (63%), 14 (88%) and 11 patients (69%), respectively. The presence of blood was not observed in any patient. The numbers of red spots and mucosal breaks were 5.5 ± 35.7 and 3.7 ± 5.1, respectively.

Efficacy of Switching from Nonselective NSAIDs to Celecoxib

Small bowel lesions were observed in 12 of 14 patients (86%) at posttreatment VCE. Similar to baseline VCE, the presence of blood was not observed in any patient. VCE findings were compared before and after treatment with celecoxib in the 14 subjects who completed this study. The proportion of patients having reddened folds, denuded areas, petechiae/red spots and mucosal breaks decreased from 71 to 50%, 64 to 50%, 93 to 79% and 71 to 36%, respectively. We performed quantitative analyses of small bowel injury in each patient before and after treatment with celecoxib. The numbers of petechiae/red spots and mucosal breaks were significantly decreased from 4.9 ± 3.5 to 1.9 ± 1.7 ($p = 0.002$; Bonferroni-adjusted $p = 0.010$) and 4.1 ± 5.4 to 0.4 ± 0.6 ($p = 0.004$; Bonferroni-adjusted $p = 0.0195$), respectively (fig. 1c, d). The scores of reddened folds and the number of denuded areas did not significantly differ before and after the treatment of celecoxib ($p = 0.031$, Bonferroni-adjusted $p = 0.157$; $p = 0.094$, Bonferroni-adjusted $p = 0.469$, respectively; fig. 1a, b). Lewis scores significantly decreased from 290 ± 248 to 92 ± 112 ($p = 0.002$; Bonferroni-adjusted $p = 0.010$; fig. 2).

Assessment of the Severity of RA

We next assessed serum and clinical markers for the disease activity of RA. There were no significant differences in Hb ($p = 0.029$; Bonferroni-adjusted $p = 0.114$), CRP ($p = 0.734$; Bonferroni-adjusted $p = 1$), MMP-3 ($p = 0.786$; Bonferroni-adjusted $p = 1$) and DAS28-CRP ($p = 0.167$; Bonferroni-adjusted $p = 0.667$) before and after treatment with celecoxib (fig. 3). We assessed serum cytokine concentrations by multiplex cytokine assay. There were no cytokines whose expressions were significantly different before and after treatment with celecoxib (table 2). Thus, we observed a significant reduction in small bowel injury by switching from nonselective NSAIDs to celecoxib after the 12 weeks of administration in patients with RA without a significant difference in the effectiveness of the medication on RA.

Discussion

Recently, VCE enabled to evaluate NSAID-induced small bowel injury, and we showed celecoxib use could reduce small bowel injury compared with nonselective NSAIDs. Goldstein et al. [16, 17] reported two randomized controlled trials using VCE in healthy volunteers and

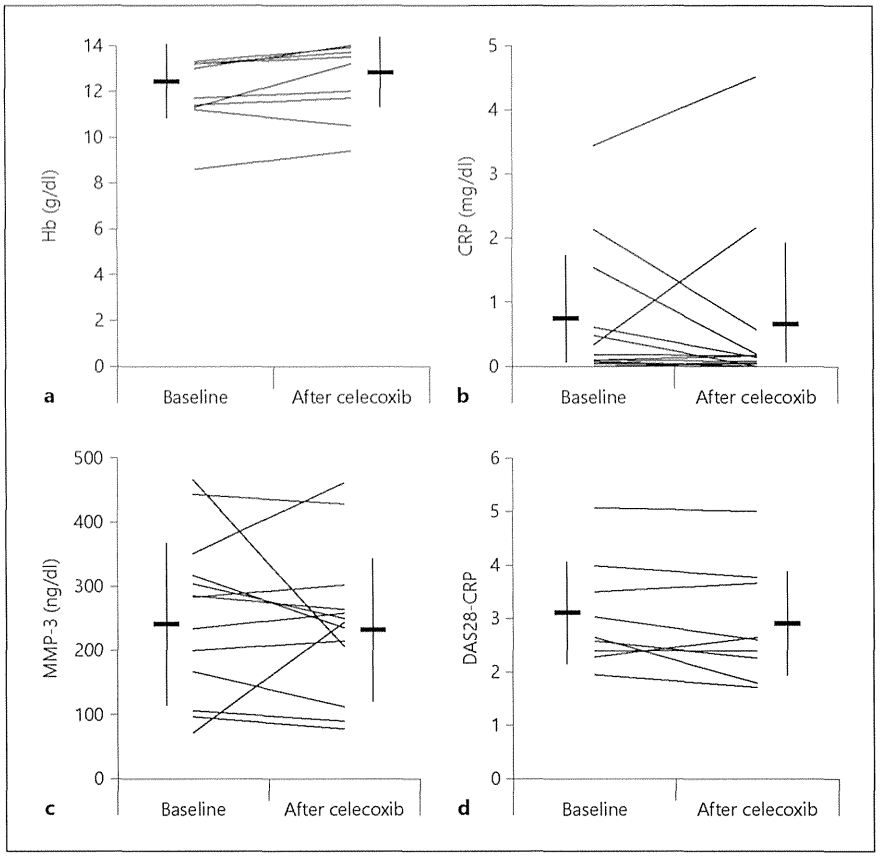

Fig. 3. Effect of conversion from nonselective NSAIDs to celecoxib on the activity of RA. Blood tests and disease activity scores were evaluated at baseline and after celecoxib treatment in 14 patients. **a** Blood Hb concentration (n = 14). **b** Serum CRP (n = 14). **c** MMP-3 (n = 14). **d** DAS28-CRP (n = 9). Bars indicate mean ± SD.

Table 1. Details of patients and treatment for RA

Age, years	59.0±11.8[a]
Sex	
Male	6 (38%)
Female	10 (62%)
Body mass index	23.5±3.9[a]
Prior NSAID therapy	
Loxoprofen	13 (81%)[b]
Diclofenac	3 (19%)[b]
Indomethacin	1 (6%)
Treatments for RA other than NSAIDs	
Corticosteroids	12 (86%)
Methotrexate	11 (69%)
Salazosulfapyridine	6 (38%)
Bucillamine	2 (13%)
Laboratory tests	
CRP, mg/dl	0.65±0.96[a]
MMP-3, ng/dl	222±135[a]
DAS28-CRP	3.2±1.1[a]
Hb, g/dl	12.6±1.6[a]

[a] Mean ± SD.
[b] One patient took a combination of loxoprofen and diclofenac.

concluded that celecoxib was effective for decreasing small bowel injury in comparison to nonselective NSAIDs. Mizukami et al. [24] also reported that celecoxib reduced small bowel injury compared with loxoprofen. Although their reports clearly showed the efficacy of celecoxib in comparison to NSAIDs for reducing small bowel injury, these reports had limitations. The duration of the celecoxib/NSAID treatment was relatively short (2 weeks), and the subjects were not diseased patients who required NSAIDs. Chan et al. [25] reported that anemia caused by small bowel injury was lower in celecoxib than diclofenac and omeprazole in patients with RA taking either medication for 6 months. However, they did not directly evaluate this small bowel damage by radiologic or endoscopic methods such as VCE. Our results revealed that celecoxib reduced the number of petechiae/red spots and mucosal breaks. Lewis scores were significantly decreased after the conversion to celecoxib. Thus, we demonstrated for the first time that small bowel injury was significantly improved by switching from NSAIDs to celecoxib in RA patients.

Table 2. Multiplex immunobead assay at baseline (B) and after celecoxib treatment (P)

Cytokine	B, pg/ml	P, pg/ml	Fold change P/B[a]	p value[b]
PDGF-BB	105,441 (31,476–1,164,451)	97,588 (46,382–1,216,712)	0.89	0.58
IL-1β	37 (15–490)	30 (15–965)	0.96	0.5
IL-1ra	1,226 (526–1,911)	1,134 (500–2,721)	1.15	0.58
IL-2	3 (0–18.79)	18 (0–45.4)	5.26	0.84
IL-4	55 (34–94)	55 (33–86)	0.99	1
IL-5	25 (9–130)	27 (6–105)	1.02	0.47
IL-6	152 (28–1,430)	92 (47–5,369)	1.96	0.95
IL-7	158 (84–351)	170 (78–296)	1.05	0.67
IL-8	246 (108–39,611)	219 (126–90,527)	1.18	0.63
IL-9	568 (51–3,923)	656 (16–9,347)	1.25	1
IL-10	132 (12–14,014)	113 (21–14,527)	1.05	0.36
IL-12	331 (64–9,501)	324 (85–5,704)	0.74	0.76
IL-13	85 (23–311)	88 (17–297)	1.12	1
IL-15	1 (0–10)	19 (0–252)	30.93	0.63
IL-17	107 (0–307)	84 (0–1,117)	1.28	0.91
Eotaxin	730 (0–6,917)	726 (0–8,988)	1.44	0.5
Basic FGF	253 (110–447)	218 (100–6,136)	2.61	0.71
G-CSF	218 (123–350)	226 (120–363)	1.05	0.81
GM-CSF	46 (0–434)	59 (0–772)	1.30	0.97
IFN-γ	2,312 (1,052–4,574)	2,721 (1,059–5,314)	1.06	0.5
IP-10	21,271 (10,152–189,971)	27,085 (12,249–216,373)	1.28	0.27
MCP-1	252 (85–451)	324 (173–1,067)	1.41	0.1
MIP-1α	58 (14–5,554)	62 (24–4,666)	0.52	0.63
MIP-1β	1,143 (471–45,475)	957 (618–22,649)	0.47	0.86
RANTES	22,134 (13,799–39,568)	22,134 (17,876–40,407)	1.01	0.88
TNF-α	624 (220–1,543)	412 (282–1,665)	0.90	1
VEGF	2,650 (581–9,218)	2,537 (656–12,726)	1.23	0.39

Values for B and P are expressed as median (range). PDGF = Platelet-derived growth factor; FGF = fibroblast growth factor; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte/macrophage colony-stimulating factor; IP-10 = IFN-inducible protein-10; MCP-1 = monocyte chemoattractant protein-1; RANTES = regulated and normal T cell expressed and secreted; VEGF = vascular endothelial growth factor.

[a] Fold change in mean cytokine level.

[b] Wilcoxon paired signed-rank test.

It has been reported that patients with RA have small bowel injury in high frequency regardless of their NSAIDs use [20]. In addition, Sugimori et al. [20] showed that patients with RA taking NSAIDs were at high risk of severe small bowel injury. Consistent with previous reports [16, 20, 21], we obtained the results that patients with RA taking NSAIDs had a high frequency of small intestinal injury. Although the proportion of RA patients who had mucosal injury was not significantly changed by switching NSAIDs to celecoxib, the severity of small bowel injury was significantly improved. In addition, the efficacy of pain control in patients with RA by celecoxib was not inferior to that of nonselective NSAIDs. These results indicate that COX-2 selective inhibition by celecoxib is beneficial for the protection of mucosal damage in the small intestine without sacrificing the efficacy of pain control in patients with RA. In spite of the absence of abdominal symptoms in most RA patients in this study, 71% of the patients with RA presented mucosal breaks by VCE and 50% presented abnormal Hb concentrations. Although it may not be necessary to switch from nonselective NSAIDs to celecoxib in all the patients, evaluation of the small intestine should be considered when the RA patients with chronic NSAID users develop persistent severe anemia but do not have diseases in the upper and lower GI tract. In such cases, switching from nonselective NSAIDs to celecoxib may be beneficial.

Maiden et al. [21] previously reported that long-term COX-2 selective agents caused small-bowel damage comparable to NSAIDs. The discrepancy in the effectiveness

of COX-2 selective agents between long- and short-term use might be caused by the fact that the majority of the subjects analyzed in their study were patients with osteoarthritis, and only 24–33% of RA patients were included. In addition, COX-2 selective agents other than celecoxib were used in 40% of the patients in their study (etoricoxib, 20%; rofecoxib, 15%; valdecoxib, 5%). Furthermore, they did not quantitatively evaluate the severity of small bowel injury. Even in their analysis, small bowel injury was observed in a lower percentage by COX-2 selective agents than by NSAIDs. Further long-term studies using celecoxib for the patients with RA are necessary to clarify this issue.

Consistent with the previous reports, we found that celecoxib did not worsen the severity of RA, including blood Hb concentration, CRP, MMP-3 and DAS28-CRP. No patients discontinued celecoxib because of a worsening of pain caused by RA. When we assessed serum cytokine concentrations by multiplex cytokine assay, which had been reported to correlate with disease activity of RA [26–28], there were no cytokines whose expressions were significantly different before and after treatment with celecoxib. These results indicate that the conversion from NSAIDs to celecoxib was tolerable without diminishing the efficacy of pain control for RA patients. We did not include patients on biologics, such as anti-TNF and anti-IL-6 receptor antibodies, because these medications might be protective for small bowel injury [29–31]. Infliximab has been reported to be protective for indomethacin-induced small bowel damage in rats [32]. Similarly, there are several reports showing the efficacy of antiulcer drugs on preventing NSAID-induced small bowel injury [33–36]. We excluded patients taking these drugs, although those were the majority of long-term NSAIDs users. On the other hand, we included patients taking gastric acid suppressants such as PPI and H2 receptor antagonist because it is still controversial whether the inhibition of gastric acid exacerbate small intestinal injury. Lansoprazole has been reported to ameliorate NSAID-induced small bowel injury in rats by inhibiting inducible nitric oxide synthase expression, while omeprazole has no effect [37, 38]. Conversely, there are some recent reports showing the opposite result that PPIs exacerbate or have no effect on NSAID-induced small intestinal injury [39–41]. Moreover, these reports concerned indomethacin-induced rat models and not humans. Additionally, because corticosteroids were reported to be either ulcerogenic [42, 43] or gastroprotective [44, 45], patients on high-dose corticosteroids were eliminated from our study. We tried to avoid medications which may affect the small intestinal injury as well as the severity of RA.

There are some limitations to this study; the sample size of the study is relatively small. This is neither a placebo-controlled study nor a crossover study. There was no control group continuing nonselective NSAIDs. Thus, we cannot completely deny the possibility that the lesions would have improved if the prior NSAIDs had been continued or switched to different nonselective NSAIDs. We did not set a washout period after the cessation of NSAIDs use because the joint pain of arthritis is not tolerable without medication with NSAIDs. The duration of medication by celecoxib in this study was relatively short. In spite of these limitations, this study clearly demonstrates that the COX-2 selective inhibitor celecoxib has a clinical benefit for the improvement of small bowel injury in RA without diminishing the efficacy of its pain control. Additional long-term and large-scale studies are necessary to clarify the role of celecoxib in small bowel injury.

In conclusion, the incidence of small bowel injury by nonselective NSAIDs is high in patients with RA. Conversion from nonselective NSAIDs to celecoxib can become a useful therapeutic option for the treatment of small bowel injury in patients with RA.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

References

▶1 Feely MG, Erickson A, O'Dell JR: Therapeutic options for rheumatoid arthritis. Expert Opin Pharmacother 2009;10:2095–2106.

▶2 Laine L: Approaches to nonsteroidal anti-inflammatory drug use in the high-risk patient. Gastroenterology 2001;120:594–606.

▶3 Gabriel SE, Jaakkimainen L, Bombardier C: Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. Ann Intern Med 1991;115:787–796.

▶4 Singh G, Rosen Ramey D: NSAID induced gastrointestinal complications: the ARAMIS perspective – 1997. Arthritis, Rheumatism, and Aging Medical Information System. J Rheumatol Suppl 1998;51:8–16.