Fig. 4. mRNA expression of IL-18 in the adrenal glands of wild-type mice. IL-18 expression levels in the Stress group and the DSS+Stress group were increased significantly compared with that in the Water group. IL-18 expression in the DSS+Stress group was increased significantly than in the DSS group. Data are shown as means ± SE; $n = 6$. $P < 0.05$ vs. the water group (*) and vs. the DSS group (#).

the DSS plus psychological stress group were increased significantly compared with the level in the control group. IL-18 expression in the DSS group was also increased compared with that in the control group. However, the increase was not significant. The increase in the DSS plus psychological stress group was significant even compared with the expression in the DSS group.

Exacerbation of DSS colitis by psychological stress was not observed without IL-18. We showed that psychological stress aggravated colitis with enhanced expression of proinflammatory cytokines and IL-18. Next, we tried to clarify whether IL-18 was involved in this process. For this purpose, we used IL-18-deficient mice. Figure 5A shows the effect of DSS and psychological stress on percent change in body weight of IL-18 knockout mice. The IL-18 knockout mice in the DSS group and the DSS plus psychological stress group showed significant weight loss compared with the weight in the water group. However, the degree of body weight loss in the DSS plus psychological stress group was almost the same, and stress-induced body weight loss was not observed in IL-18 knockout mice, suggesting that psychological stress-induced aggravation of colitis was IL-18-dependent. Figure 5B shows the effect of psychological stress in IL-18 knockout mice on DSS colitis determined by colonic length. Colonic lengths in the DSS group and the DSS plus psychological stress group were decreased significantly compared with that in the water group. Shortening of the colon was almost the same in the DSS group and the DSS plus psychological stress group, and psychological stress-induced aggravation of DSS colitis was not observed. Figure 6 shows a representative H & E section of the distal colon and grade of colonic inflammation in each group of IL-18 knockout mice. There were few inflammatory cells and intact crypts in the colonic mucosa of IL-18 knockout mice in the water group. On the other hand, mild colitis was observed
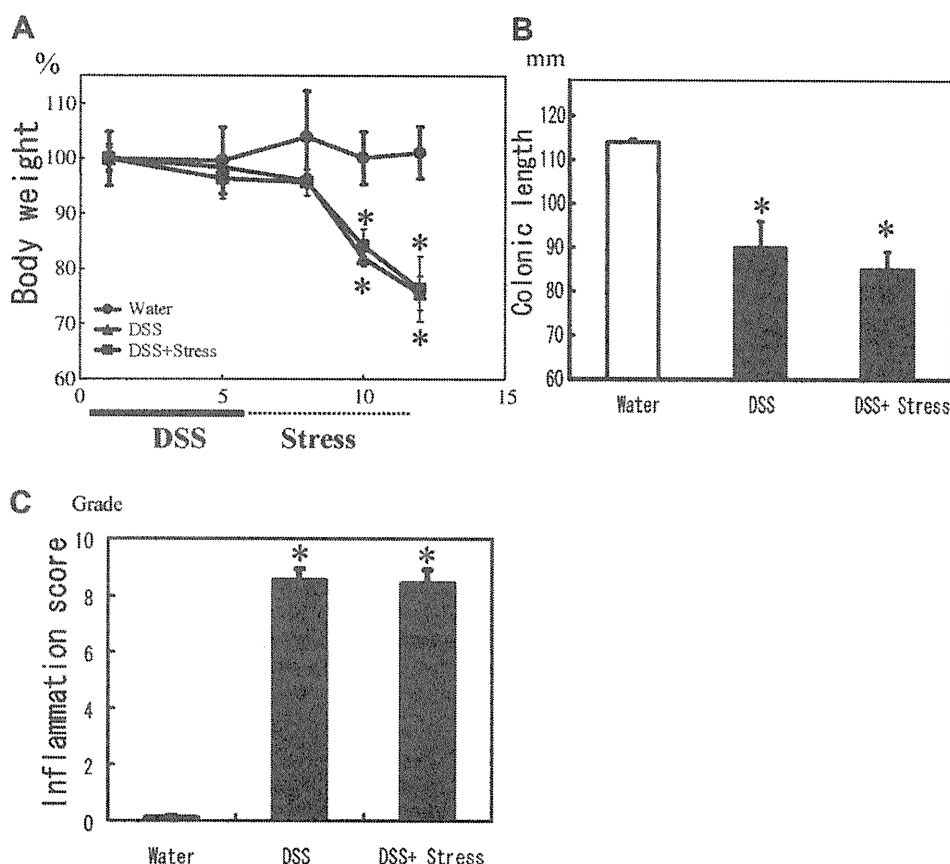

Fig. 5. Effects of DSS and psychological stress on percent change of body weight (A), colonic length (B), and grade of mucosal inflammation (C) in each group of IL-18 knockout mice. Induction of psychological stress in IL-18 knockout mice did not aggravate DSS-induced colitis. Data are shown as means ± SE; $n = 5$. *$P < 0.05$ vs. the water group.
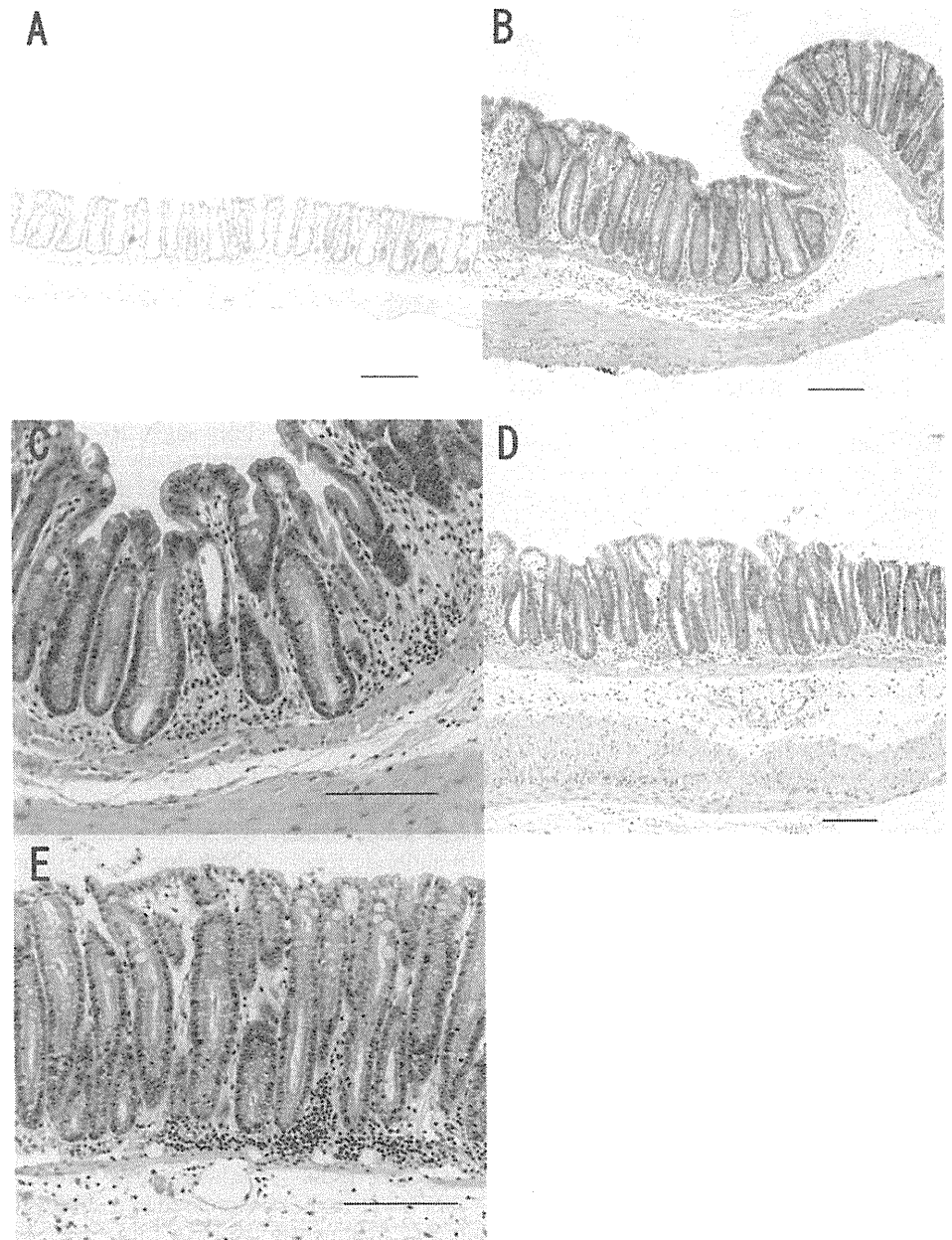
Fig. 6. Effects of DSS and psychological stress on representative H & E sections of the distal colon in each group of IL-18 knockout mice. *A*: a control group administered only water. *B* and *C*: a group administered DSS. *D* and *E*: a group administered DSS and exposed to psychological stress. Induction of psychological stress in IL-18 knockout mice did not aggravate DSS-induced colitis. Bars indicate 100 μm. *A*, *B*, and *D*: ×10 objective. *C* and *E*: ×20 objective.

in IL-18 knockout mice treated with DSS, and the colitis was characterized by increased inflammatory cells, a decrease in the number of crypts, and an increase in thickness of submucosal and muscularis layers. However, induction of psychological stress in IL-18 knockout mice did not enhance DSS-induced colitis, and the activity of colitis remained mild. Crypt formation was preserved in the DSS plus stress group of IL-18 knockout mice but had completely disappeared in the same treatment group of wild-type animals (Fig. 1*C*). Figure 5*C* shows the grade of colonic inflammation in each group of IL-18 knockout mice. Histological damage scores in the DSS group and the DSS plus psychological stress group were increased significantly compared with that in the water group. The IL-18 knockout mice in the DSS plus psychological stress group did not show further aggravation compared with the

histological damage score in the DSS group of IL-18 knockout mice. We showed that psychological stress-induced aggravation of colitis was accompanied by an increase in TNF-α. We next clarified whether psychological stress increased TNF-α in DSS-induced colitis in the absence of IL-18. Figure 7 shows relative mRNA expression of TNF-α in the colonic mucosa of each group of IL-18 knockout mice. Interestingly, psychological stress-induced enhancement of TNF-α expression in DSS-treated mice was not observed in IL-18 knockout mice, suggesting that TNF-α is involved in stress-induced immune imbalance.

Effect of blocking of IL-18 by anti-IL-18 antibody on psychological stress-induced aggravation of DSS colitis. To further evaluate the role of IL-18 in psychological stress-induced aggravation of DSS colitis, we divided wild-type mice into the
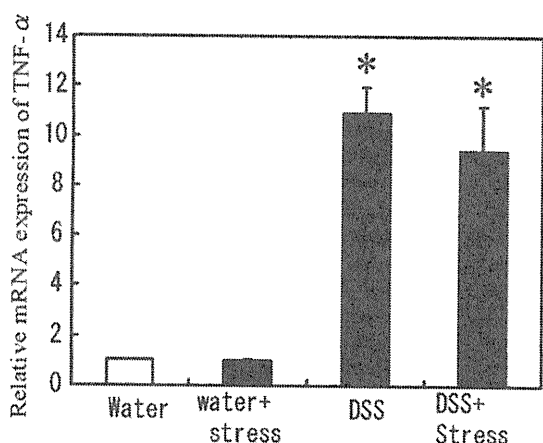
Fig. 7. mRNA expression of TNF-α in the colonic mucosa of each group of IL-18 knockout mice. Stress-induced enhancement of TNF-α expression in DSS-treated mice was not observed in IL-18 knockout mice. Data are shown as means ± SE; $n = 5$. *$P < 0.05$ vs. the water group.

following three groups: DSS plus psychological stress with IL-18 antibody treatment group, DSS plus psychological stress with control antibody treatment group, and water-administered control group. We treated mice with antibodies 3 h before exposure to psychological stress from *day 6* to *day 10*. In the DSS plus psychological stress with control antibody treatment group, body weight decreased during DSS treatment (*day 1* to *day 6*) and continued to decrease even during the stress exposure period (*day 6* to *day 11*). On the other hand, in the DSS plus psychological stress with anti-IL-18 antibody treatment group, body weight started to increase after anti-IL-18 antibody treatment, and body weight loss was significantly less than that in the DSS plus psychological stress with control antibody treatment group (Fig. 8A). Shortening of colonic length was also less in the DSS plus psychological stress with IL-18 antibody treatment group than in the DSS plus psychological stress with control antibody treatment group (Fig. 8B). Grade of colonic inflammation was also less in the DSS plus psychological stress with IL-18 antibody treatment group than in the DSS plus psychological stress with control antibody treatment group (Fig. 8C). The level of expression of proinflammatory cytokines, TNF-α and IL-6, was also lower in the DSS plus psychological stress with IL-18 antibody treatment group than in the DSS plus psychological stress with control antibody treatment group (Fig. 8, *D* and *E*). The number of infiltrating cells in the colonic mucosa was also less in the DSS plus psychological stress with IL-18 antibody treatment group than in the DSS plus psychological stress with control antibody treatment group (Fig. 8F). These results directly showed that neutralization of IL-18 ameliorated psychological stress-induced aggravation of DSS colitis.

TNF-α increased expression levels of IL-18 receptor and proinflammatory cytokines in a colonic epithelial cell line. Finally, we investigated whether epithelial cells expressed IL-18, IL-18 receptor, and proinflammatory cytokines that are known to be induced through the NF-κB pathway (IL-8 and ICAM-1). In addition, we investigated how the cells were affected by TNF-α and/or IL-18 treatments. LS-174T cells expressed mRNAs of IL-18, IL-18 receptor, IL-8, and ICAM-1 in the control condition. TNF-α, which is known to activate the

NF-κB pathway, significantly increased the expression levels of IL-8 and ICAM-1 mRNAs, suggesting that this cell line is useful for evaluating the effect of IL-18 on expression of proinflammatory cytokines. However, treatment with IL-18 alone did not enhance the expression of IL-8 or ICAM-1. Because TNF-α increased IL-18 receptor expression, we treated cells with both TNF-α and IL-18 to highlight the role of IL-18. However, addition of IL-18 to TNF-α did not enhance IL-8 or ICAM-1 expressions, suggesting that proinflammatory cytokines from epithelial cells were not involved in IL-18-induced aggravation of colitis (Fig. 9).

DISCUSSION

Psychological stress is an environmental factor that has long been suggested to contribute to the pathophysiology of IBD. A recent study has suggested that several stressors can increase the rate of relapse in patients with IBD (21). It has been shown that captivity stress and readjustment to a novel social environment cause spontaneous colitis in cotton-topped tamarins (8, 11). In the present study, we showed for the first time by using a mouse model of colitis that addition of psychological stress to colonic inflammation caused aggravation of colitis. Mice were exposed to various emotional conditioned stimuli (psychological stress) by watching and hearing the stress responses of physically stressed mice that received electric shock. This model made it possible to clarify the mechanism of psychological stress-induced aggravation of colitis by using genetically engineered mice. We treated mice with psychological stress after the DSS drinking period to avoid an affect of psychological stress on the quantity of DSS consumption. In addition, we treated mice with a lower concentration of DSS for induction of colitis to distinguish the effect of psychological stress on colitis. Almost all parameters, including body weight loss, shortening of colonic length, and histological inflammation, were aggravated by psychological stress.

The mechanism of modulation of stress to the colonic immune system is not clear. However, several pathways have been suggested: *1)* the HPA axis pathway, 2) the systemic nervous system axis pathway, and *3)* the opioid receptor system pathway. We paid attention to the HPA axis in these pathways. We focused on IL-18 because *1)* IL-18 mRNA is expressed in response to a stressor in the adrenal gland through the HPA axis (5), 2) stress-induced IL-18 enhances production of proinflammatory cytokines (40), and *3)* IL-18 is involved in the pathophysiology of stress-induced histamine-dependent gastric injury (39). In this study, we treated mice with an anti-IL-18 neutralizing antibody during the period of exposure to stress, which was after DSS drinking treatment. Anti-IL-18 antibody significantly ameliorated stress-induced aggravation of colitis compared with the control antibody-treated group. In addition, stress-induced aggravation of colitis was not observed in IL-18$^{-/-}$ mice. Taken together, these results suggested that stress-induced aggravation of colitis was IL-18-dependent. In this study, psychological stress enhanced expression of IL-18 in the adrenal gland, suggesting that the HPA axis pathway was activated by communication box-induced psychological stress. Interestingly, enhanced expression of IL-18 was observed not only in the adrenal gland but also in the colonic mucosa. Recently, it has been reported that isolation stress enhanced expression of IL-18 in the murine rectum (30).
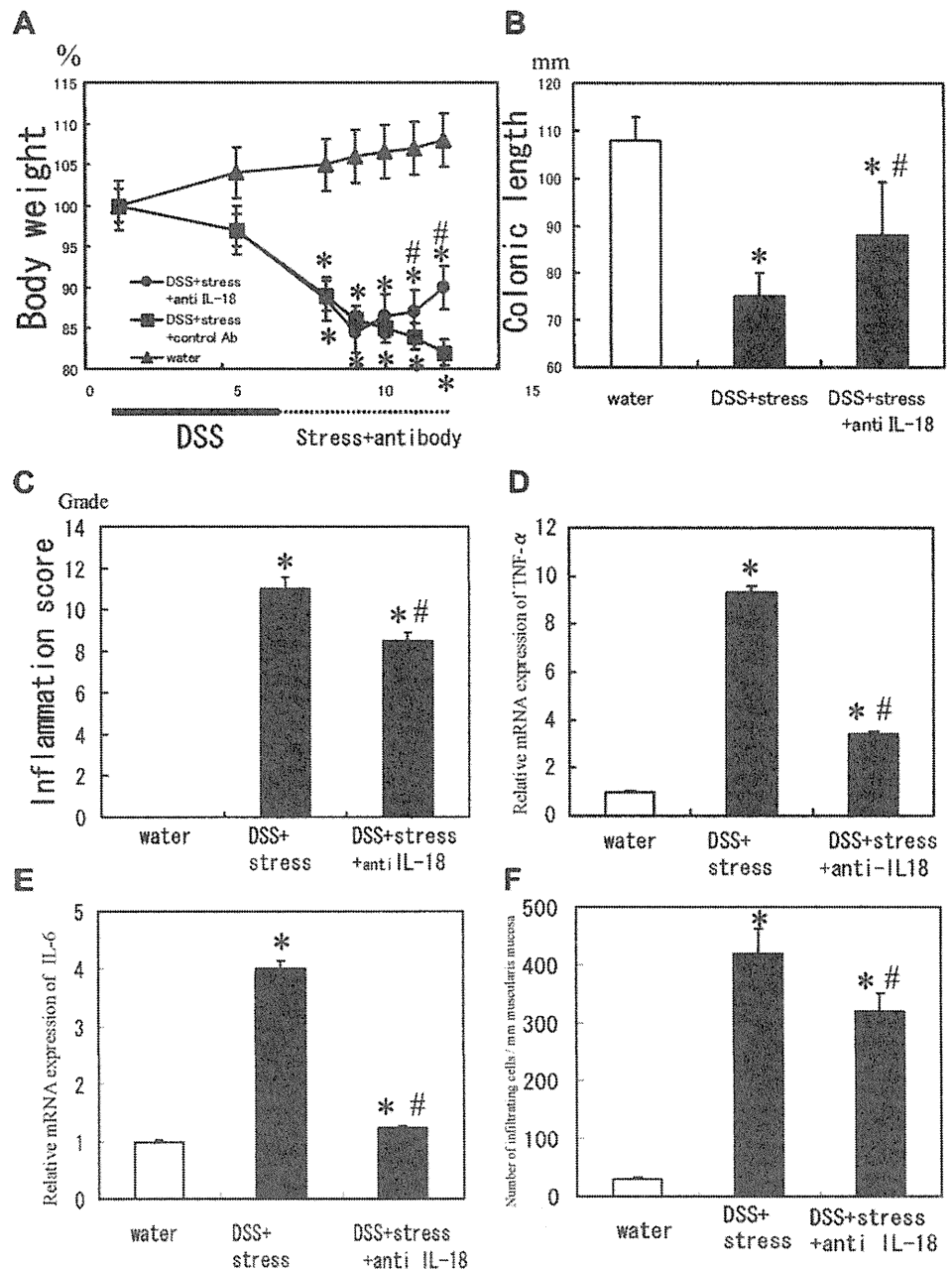
Fig. 8. Effect of anti-IL-18 antibody on psychological stress-induced aggravation of colitis assessed by percent change of body weight (A), colonic length (B), grade of mucosal inflammation (C), mRNA expression of TNF-α (D), mRNA expression of IL-6 (E), and no. of cells infiltrating the colonic mucosa (F). DSS+Stress+anti-IL-18, a group administered anti-IL-18 antibody and DSS and exposed to psychological stress. Anti-IL-18 antibody treatment ameliorated stress-induced aggravation of colitis. Data are shown as means ± SE; $n = 5$. $P < 0.05$ vs. the control (*) and vs. the DSS+Stress group (#).

Kanai et al. (16) and Siegmund et al. (41) reported differential IL-18 promoter usage in the adrenal gland and immune cells with adrenal gland-specific expression of IL-18 mRNA by ACTH. It has been reported that both epithelial cells and macrophages expressed IL-18 in the colonic mucosa of murine colitis (16, 41). Because we did not use organ-specific conditional knockout IL-18 mice, it is not known from which organ and which kind of cells that IL-18 was responsible for aggravation of stress-induced colitis. The source of IL-18-producing cells in the colonic mucosa and the mechanism of regulation have yet to be clarified. However, IL-18 induction only by psychological stress in the rectal mucosa suggests that the large intestine is the vulnerable organ for stress-induced aggravation of inflammatory response. To clarify possible involvement of IL-18 in epithelial cells in the stress-induced immune response,

we investigated the expression of IL-18, IL-18 receptor, and proinflammatory cytokines that are known to be induced through the NF-κB pathway in an epithelial cell line in vitro. Epithelial cells expressed both IL-18 and IL-18 receptor. However, treatment of epithelial cells with IL-18 did not enhance the expression of IL-8 or ICAM-1. These results suggested that epithelial cells may be a source of IL-18 but unlikely a target for its action.

In our study, expression of TNF-α was increased by DSS treatment, and psychological stress further increased TNF-α expression. The absence of IL-18 completely blocked the psychological stress-induced increase in TNF-α expression. IL-18 enhances the production of both Th1 and Th2 cytokines (7, 14, 28, 29). IL-18 aggravates TNF-α-induced hepatic injury in mice (48). It has been reported that IL-18-induced TNF-α
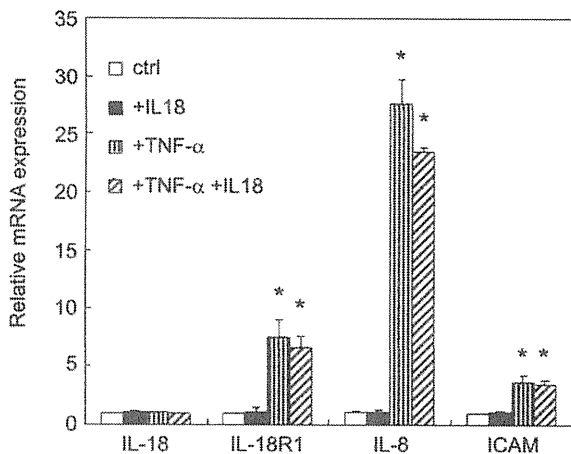
Fig. 9. Effect of TNF-α and IL-18 on expressions of IL-18, IL-18 receptor-1 (IL-18R1), IL-8, and intercellular adhesion molecule (ICAM)-1 in the epithelial cell line LS174T. mRNA levels were detected after stimulation with TNF-α (20 ng/ml), IL-18 (20 ng/ml), and TNF-α + IL-18 (20 ng/ml + 20 ng/ml) for 6 h. TNF-α enhanced expressions of IL-18R1, IL-8, and ICAM-1 significantly. Data are shown as means ± SE; $n = 5$. *$P < 0.05$ vs. control.

expression was observed in $CD3^+/CD4^+$ cells but not in $CD14^+$ macrophage lineage cells and that this induction was inhibited by IL-10 (34). It is generally accepted that a relative decrease in IL-10 is responsible for the pathogenesis of IBD. Thus, stress-induced IL-18 production might play a proinflamatory role synergistically with a decrease in IL-10.

Previously, Kanai et al. (15, 16) showed the involvement of IL-18 in murine colitis. In our study also, DSS treatment alone enhanced expression of IL-18 in the murine colon. Because we induced mild colitis, the level of IL-18 expression induced by DSS treatment was comparable to that induced by psychological stress alone. It is possible that DSS treatment itself was a stressor to induce IL-18. Alternatively, it is possible that IL-18 was upregulated by another cytokine indirectly. In this study, the degree of DSS-induced colitis in wild-type mice and that in IL-18$^{-/-}$ mice was comparable. However, stress-induced aggravation was completely blocked in the absence of IL-18. In addition, systemic inhibition by an IL-18 neutralizing antibody ameliorated stress-induced aggravation of colitis. Collectively, the results suggest that IL-18 is involved in the pathophysiology of proinflammatory response in colonic inflammation under psychologically stressful conditions.

GRANTS

DISCLOSURES

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Correlation between endocytoscopy and conventional histopathology in microstructural features of ulcerative colitis

Rieko Bessho · Takanori Kanai · Naoki Hosoe ·
Taku Kobayashi · Tetsuro Takayama · Nagamu Inoue ·
Makio Mukai · Haruhiko Ogata · Toshifumi Hibi

Abstract

Background Routine diagnosis of the histopathological activity of ulcerative colitis (UC) requires multiple biopsy samples, and an endocytoscopy system (ECS) provides real-time ultra-magnifying microscopic imaging in vivo.
Methods We have established an ECS score (ECSS) to determine a histopathological activity index of UC. Fifty-five UC patients (mean age 40.7 years; 67% men) were enrolled. A super-magnifying ECS with magnification $450\times$ was used, and sample biopsies were obtained. Matts' histopathological grade was determined, to evaluate disease severity, by two pathologists, with consensus. The ECSS of UC was independently determined by at least two investigators, with consensus. In total, 76 pairs of ECSS and Matts' histopathological grades were independently acquired. To validate the ECSS, inter-observer agreement between three endoscopists, with consensus, and another endoscopist, was calculated as the kappa value. We also evaluated the correlation between the ECSS and Matts' histopathological grade, and between the conventional Matts' endoscopic grade and Matts' histopathological grade.

Results The ECSS of UC intestinal mucosa, i.e., the sum of the indices for shape (0–3) and distance between crypts (0–2), and the visibility of superficial microvessels (0–1), showed a strong correlation with Matts' histopathological grades ($\rho = 0.713$, $P < 0.001$); as well, there was a strong correlation between the conventional Matts' endoscopic grade and Matts' histopathological grade ($\rho = 0.694$, $P < 0.001$). Furthermore, the ECSS showed high reproducibility ($\kappa = 0.79$, 95% confidence interval [CI] 0.71–0.87).
Conclusions Our novel ECSS has good predictive value for the histopathological activity of UC.

Keywords Endocytoscopy · Ulcerative colitis · Mucosal inflammation · Histopathology

Introduction

Two devices are currently available that allow in vivo microscopic inspection of the microstructural mucosal features of the gastrointestinal tract: confocal laser endomicroscopy (CLE) (Pentax, Tokyo, Japan, and Mauna Kea Technologies, Paris, France) [1–6] and an endocytoscopy system (ECS) with an ultra-magnification light microscopy device (Olympus Medical Systems, Tokyo, Japan) [1, 7–10]. There are two types of each device, probe-based and integrated-scope types [6, 11]. These devices can facilitate the distinguishing of neoplastic from non-neoplastic lesions [3, 4, 7, 8, 11, 12], and also in classifying the severity of inflammatory lesions [9, 13] histopathologically. For CLE, several groups have recently reported its use for the detection of the microstructural features of the mucosa in ulcerative colitis (UC) patients [12, 13], and one group attempted to identify a correlation

R. Bessho · T. Kanai · T. Kobayashi · T. Takayama ·
T. Hibi (✉)
Division of Gastroenterology and Hepatology,
Department of Internal Medicine, Keio University School
of Medicine, Tokyo 160-8582, Japan
e-mail: thibi@sc.itc.keio.ac.jp

N. Hosoe · N. Inoue · H. Ogata
Center for Diagnostic and Therapeutic Endoscopy,
School of Medicine, Keio University, Tokyo, Japan

M. Mukai
Division of Diagnostic Pathology, School of Medicine,
Keio University, Tokyo, Japan

between classification by CLE and paired histological sample findings [13]. However, no such studies have been conducted for ECS. ECS can provide a real-time image, as with light microscopy, and facilitate rapid diagnosis [10]. The aims of the present study were: (1) to develop a new UC scoring system based on ECS and (2) to validate the pathological and clinical utility of this scoring system.

Methods

Enrolled patients and ECS procedure

Fifty-five patients with a confirmed diagnosis of UC were prospectively enrolled in this study from April 2009 to April 2010. Written informed consent was obtained from all subjects, and the study was approved by the ethics committee of Keio University Hospital. Six experienced endoscopists (R. B., T. Ka., N. H., T. Ko., N. I., and H. O.) performed total colonoscopy with a conventional colonoscope (CF-Q260AI; Olympus Medical Systems). If patients agreed to participate in this study, the ECS (ECS, CF-Y0001; Olympus Medical Systems) was used to obtain more sensitive, ultra-magnified images of the rectal area. Our ECS was an integrated scope-type ultra-magnifying system and could be switched from conventional and magnifying views to super-magnifying, using a button located at the top of the endoscope. The ECS was used at the representative part of the rectal area that had been detected by conventional and magnifying endoscopy. When differences in endoscopic activities were observed, multiple ECS images and biopsy samples were taken. The rectal mucosa was washed with an excess of water plus simethicone, and stained with 10 mL of 1% methylene blue solution. The excess stain was rinsed off to avoid over-staining the cells. It takes a few minutes to perform dye staining, and approximately 10–20 min to observe the surface of the lesion with the ECS [7]. Ultimately, a targeted biopsy was performed as accurately as possible for histological analysis of the same sites. All biopsy specimens were fixed in 10% formalin and embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin (H&E). Histological examination and scoring were performed by two experienced pathologists, with consensus (between five different pathologists and M. M.).

Scoring system

All reviewers were blinded to the clinical and histological backgrounds of the ECS images. First, 20 ECS pictures were reviewed, and all items relevant to the pathological features of UC were collected by one endoscopist (R. B.). The scoring system thereby created and set by this

endoscopist was called the ECS score (ECSS). Then 20 ECS pictures were scored by two experienced endoscopists (T. Ka, N. H.), upon reaching agreement. Pictures other than these were reviewed and scored by three endoscopists (R. B., T. Ka., and N. H.), also in agreement.

To validate the ECSS, inter-observer agreement between three endoscopists (R. B., T. Ka., and N. H.), with consensus, and another endoscopist, was calculated as the kappa value. Next, we evaluated the correlation between the ECSS and Matts' histopathological grade, and that between the conventional Matts' endoscopic grade and Matts' histopathological grade. Furthermore, to assess the clinical utility of the ECSS, correlations between the ECSS and clinical activity factors [C-reactive protein (CRP) level and stool frequency] were evaluated. These data were collected from medical records.

Statistical analysis

Statistical analysis was performed using PASW version 17 software (IBM, Tokyo, Japan). Statistical correlations between two groups were determined using Spearman's rank correlation coefficient. Inter-observer agreements were assessed with kappa statistics. Kappa values were

Table 1 Profiles of enrolled patients

Total number of patients	55
Age (years)	40.7 (15–69)
Male	37 (67.3%)
Disease duration (years)	8.6 (0.5–30)
Type of UC	
Total colitis	23
Left-sided	20
Proctitis	12
Clinical course	
Relapsing–remitting type	45
Chronic continuous type	4
One attack only	6
Treatment	
5-ASA	M/F
SASP	12/8
Mesalazine	37/8
Prednisolone	1/22
6-MP	7/1
AZA	2/2
Tacrolimus	0/1
CAP	1/6
No medication	4/0

UC ulcerative colitis, *5-ASA* 5-aminosalicylic acid, *SASP* salazosulfapyridine, *6-MP* 6-mercaptopurine, *AZA* azathioprine, *CAP* cell apheresis, *M* male, *F* female

Fig. 1 Representative series of conventional endoscopic, ECS, and histopathological images. A–C Case 1: a patient in clinical remission (46 years, female). D–F Case 2: a patient with active-stage ulcerative colitis (UC) (42 years, female). A, D endoscopic images. B, E H&E, ×400. C, F ECS, ×450
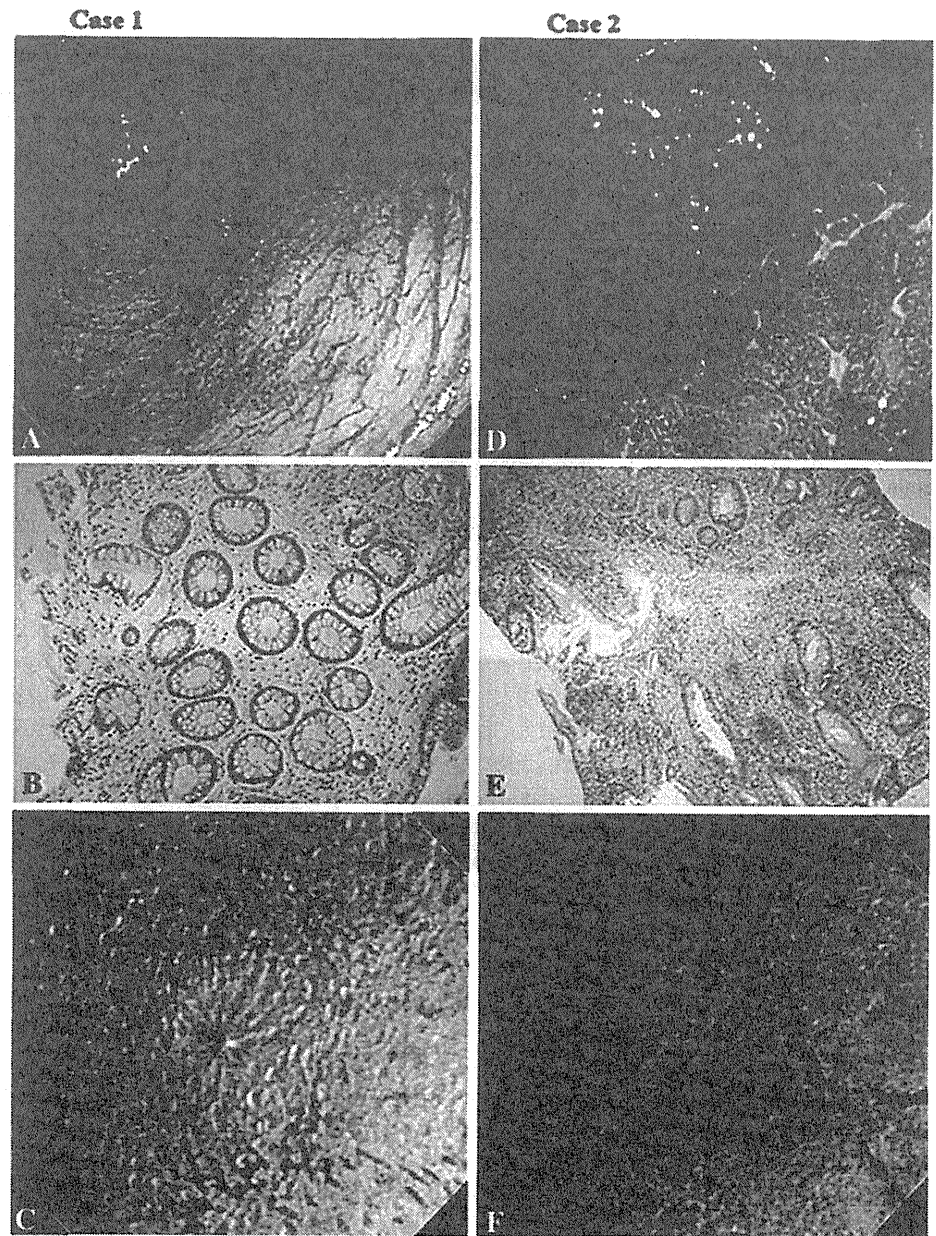
interpreted as follows: absence of agreement 0, slight agreement <0.20, fair agreement 0.21–0.40, moderate agreement 0.41–0.60, substantial agreement 0.61–0.80, and almost perfect agreement >0.81, as proposed by Landis and Koch [14].

Results

Patient demographics and characteristics are shown in Table 1. The number of enrolled patients was 55, and clinical activity was regarded as remission or mild in 51 patients and moderate in 4. When differences in endoscopic activities were observed in the rectum of the same patient, multiple ECS images and biopsy samples were taken. In total, 76 ECS images were obtained.

We selected three items from the first 20 ECS images. ECSS-A indicates the shape of crypts: 0 normal round; 1 oval, indicating possible crypt distortion; 2 irregular, indicating severe crypt distortion and destruction; and 3 not recognizable, indicating extensive crypt destruction (Fig. 2A). ECSS-B indicates the distance between neighboring crypts: 0 normal, three or more crypts are observed in a visual field; 1 intermediate, $2 \leq$ crypts < 3 in a visual field; and 2 elongated, <2 crypts in a visual field (Fig. 2B). ECSS-C indicates the visibility of superficial microvessels: 0 not visible, and 1 visible (Fig. 2C). Even in normal rectal mucosa, ECS occasionally detects microvessels. ECSS-C

Fig. 2 Endocytoscopy system score (ECSS). Three indices were adopted for the ECSS. ECS score A (ECSS-A): shape of crypts, 0 normal round; 1 oval, indicating possible crypt distortion; 2 irregular, indicating severe crypt distortion and destruction; and 3 unrecognizable, indicating extensive destruction. ECSS-B indicates the distance between neighboring crypts: 0 normal, three or more crypts in a visual field; 1 intermediate, $2 \leq$ crypts < 3 in a visual field, with infiltrating cells in the lamina propria (LP); and 2 elongated, fewer than 2 crypts in a visual field. ECSS-C indicates the visibility of superficial microvessels: 0 not visible, and 1 visible. The total ECSS is the sum of ECSS-A, B, and C (minimum 0, maximum 6)
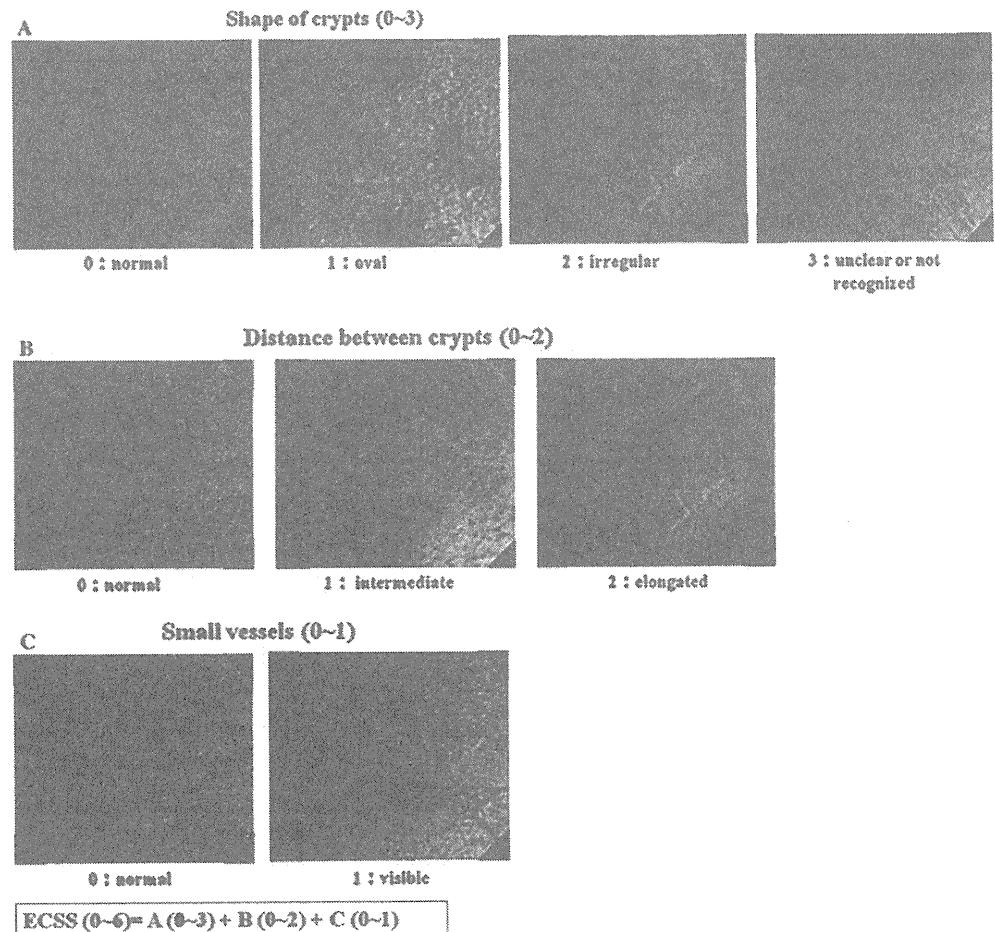

$$ECSS\,(0{\sim}6) = A\,(0{\sim}3) + B\,(0{\sim}2) + C\,(0{\sim}1)$$

Table 2 Inter-observer agreement for each evaluation item and total ECSS

	κ value	95% CI	P value
ECSS-A (shape)	0.73	0.61–0.85	<0.001
ECSS-B (distance)	0.52	0.34–0.70	<0.001
ECSS-C (vessels)	0.63	0.45–0.81	<0.001
Total ECSS	0.79	0.71–0.87	<0.001

ECSS endocytoscopy system score, *CI* confidence interval

Table 3 Correlation between conventional Matts' endoscopic grade and Matts' histopathological grade

	Matts' histopathological grade					
	1	2	3	4	5	Total
Matts' endoscopic grade						
1	12	1	0	0	0	13
2q	30	8	2	1	0	41
2a	2	0	12	0	0	14
3	0	2	2	1	2	7
4	0	0	0	0	1	1
Total	44	11	16	2	3	76

Spearman rank correlation coefficient $|r| = 0.694$

visible vessels were defined as superficial and dilated microvessels. Total ECSS is the sum of ECSS-A, B, and C (minimum 0, maximum 6). As shown in Fig. 1, a representative UC patient in the remission stage (Case 1: 46 years, female) showed almost normal mucosa except for a slightly unclear vascular pattern by conventional colonoscopy (Fig. 1A) and regular crypts with strong staining on H&E pictures (Fig. 1B). In contrast, a representative UC patient in the active stage (Case 2: 42 years, female) showed diffuse inflammation with mucosal erythema, erosion, and purulent mucus by conventional colonoscopy (Fig. 1D) and sparse, irregular crypts and marked infiltration of mononuclear cells in the lamina propria (LP) with

H&E staining (Fig. 1E). The ECS images of these two cases corresponded to the histological H&E-stained images (Fig. 1C, F). In addition, microvessels were visible in the ECS image in the patient in the active stage of UC.

To assess the reproducibility of the ECSS, kappa values were calculated. As shown in Table 2, moderate to substantial agreements were recognized for each item. Furthermore, substantial agreements between different endoscopists were observed for the total ECSS. Before

Table 4 Correlations of ECSS-A, -B and -C with Matts' histopathological grade

	Matts' histopathological grade						$|r|$[a]
	1	2	3	4	5	Total	
Shape							
0	32	6	2	1	0	41	0.568
1	12	5	8	0	1	26	
2	0	0	5	0	1	6	
3	0	0	1	1	1	3	
Distance							
0	42	8	3	0	0	53	0.745
1	2	2	6	1	2	13	
2	0	1	7	1	1	10	
Vessels							
0	44	7	8	1	0	60	0.643
1	0	4	8	1	3	16	

[a] Spearman rank correlation coefficient

Table 5 Correlation between ECSS and Matts' histopathological grade

	Matts' histopathological grade					
	1	2	3	4	5	Total
ECSS						
0	31	6	0	0	0	37
1	12	1	4	1	0	18
2	1	1	2	0	0	4
3	0	2	3	0	1	6
4	0	1	3	0	1	5
5	0	0	4	0	0	4
6	0	0	0	1	1	2
Total	44	11	16	2	3	76

Spearman rank correlation coefficient $|r|$ = 0.713

investigating the possible application of the ECSS for assessing the histopathological disease activity of UC, we evaluated the correlation between the conventional Matts' endoscopic grade and Matts' histopathological grade, as shown in Table 3. Consistent with previous reports, we found a significant correlation between the two (Spearman's $\rho = 0.694$, $P < 0.001$), although Matts' endoscopic grade (2q) tended to correspond to a broad range of Matts' histopathological grades. Next, we examined whether each ECSS index (ECSS-A, B, and C) correlated with Matts' histopathological grades (Table 4). All were found to show good correlations, with ECSS-B, the indicator of the distance between neighboring crypts, showing the strongest correlation (ECSS-A, $\rho = 0.568$, $P < 0.001$; ECSS-B, $\rho = 0.745$, $P < 0.001$; ECSS-C, $\rho = 0.643$, $P < 0.001$). Finally, we assessed the total ECSS as an indicator of UC histopathological disease activity. As shown in Table 5, there was a strong correlation between the ECSS and Matts' histopathological grades ($\rho = 0.713$, $P < 0.001$).

Correlations between the ECSS and clinical activity factors (CRP and stool frequency) were evaluated. The ECSS and stool frequency showed a weak correlation ($\rho = 0.303$, $P = 0.03$). There was no significant correlation between the ECSS and CRP.

Discussion

This is the first study to show the potential applicability of a newly developed ECS scoring system for the assessment of the histopathological disease activity of UC. First, we confirmed that the ECSS had a high kappa value, i.e., that the ECSS showed high reproducibility. The ECSS involves only three evaluation items, and each has four or fewer categories. This simple process may have contributed to the high inter-observer agreement. Next, we demonstrated a good correlation between the ECSS and Matts' histopathological grade; as well, we demonstrated a good correlation between the conventional Matts' endoscopic grade and Matts' histopathological grade. The distance between neighboring crypts (ECSS-B) ($\rho = 0.745$, $P < 0.001$) was the most reliable of the three indices. Furthermore, other items also showed significant correlations with Matts' histopathological grade. The ECSS is comprised of only three items. It does not allow assessment of conventional histopathological items, such as inflammatory cell infiltration. Thus, the ECSS is not a substitute for routine conventional histopathological examination in the evaluation of UC, but could serve as a simple surrogate for this evaluation.

Employing an approach similar to that used in the present study, Li et al. [13] have shown the benefits of classifying the histopathological activity of UC using CLE. They classified CLE findings based on crypt architecture, microvascular alteration, and fluorescein leakage into

crypts. They also analyzed the correlation between each classified item and the histological index, divided into two categories (Geboes index). On the other hand, we analyzed the correlations between three ECSS items (A, B, C) and Matts' histopathological grade, divided into five categories. Furthermore, we confirmed a strong correlation between the ECSS and Matts' histopathological grade. Therefore, the ECSS is an excellent predictor of the histopathological activity of UC.

Most clinical studies reported to date have used a CLE integrated into the distal tip of a conventional upper endoscope (iCLE: EG-3870CIK; Pentax) or a colonoscope (EC-3870CILK; Pentax) [6]. A smaller number of studies used a probe-based CLE (pCLE) (Mauna Kea Technologies) inserted through the accessory channel of a traditional endoscope [6]. Similar to the classification of CLE, ECS is classified as probe-based ECS (pECS) and integrated-scope type ECS (iECS) [11]. We used an iECS which could be switched from conventional and magnifying views to super-magnifying using a button located at the top of the endoscope. iECS is very useful in that a single scope can obtain images ranging from conventional to super-magnified.

Confocal laser endomicroscopy based on tissue fluorescence uses local and/or intravenous contrast agents and generates images [6]. ECS observation also requires pretreatment with methylene blue or toluidine blue staining [11]. In the present study, the additional time required for ECS observation was approximately 20 min. In other words, with an additional ECS procedure, we were able to predict the histopathological activity of UC.

We found no correlations between the ECSS and clinical activity. There was a weak correlation ($\rho = 0.303$, $P = 0.03$) between the ECSS and stool frequency. These results were attributed to small sample size and bias favoring the enrollment of patients with relatively mild disease activity. To assess the clinical efficacy of the ECSS, further clinical trials with a larger sample will be needed.

In conclusion, our newly developed ECSS is simple to perform and the data obtained provide a good prediction of the histological activity of UC. To confirm the clinical and histopathological usefulness of the ECSS, further clinical study with a larger sample size is needed.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Small bowel injury induced by selective cyclooxygenase-2 inhibitors: a prospective, double-blind, randomized clinical trial comparing celecoxib and meloxicam

Yuji Maehata · Motohiro Esaki · Toshibumi Morishita ·
Shuji Kochi · Shingo Endo · Kentaro Shikata ·
Hiroyuki Kobayashi · Takayuki Matsumoto

Abstract

Background Selective cyclooxygenase (COX)-2 inhibitors are less harmful to the small bowel mucosa than non-selective anti-inflammatory drugs. We aimed to compare the severity of small bowel mucosal injury in healthy volunteers induced by two selective COX-2 inhibitors, celecoxib and meloxicam, in a randomized, double-blind trial, using capsule endoscopy (CE).

Methods Twenty-nine healthy subjects were randomized to take either celecoxib (200 mg twice daily) or meloxicam (10 mg once daily) for 2 weeks. The incidence and the number of small bowel mucosal injuries (bleeding, ulcers, and erosions) observed by CE were compared between the two groups.

Results The overall incidence of small bowel mucosal injury was not different between the celecoxib group (6 of 14 subjects, 42.9%) and the meloxicam group (4 of 15 subjects, 26.7%, $P = 0.45$). In subjects with positive CE findings, the number of ulcers was greater in the meloxicam group than in the celecoxib group ($P = 0.02$), while such a trend was not found with regard to erosions ($P = 0.52$). The distribution of mucosal lesions within the small bowel was similar in the two groups.

Conclusions Selective COX-2 inhibitors are not completely safe for the small bowel. The mucosal lesions may be less severe with celecoxib than with meloxicam.

Y. Maehata (✉) · M. Esaki · T. Morishita · T. Matsumoto
Department of Medicine and Clinical Science,
Graduate School of Medical Sciences, Kyushu University,
3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: ymaehata@intmed2.med.kyushu-u.ac.jp

S. Kochi · S. Endo · K. Shikata · H. Kobayashi
Division of Gastroenterology, International University of Health
and Welfare Fukuoka Sanno Hospital, Fukuoka, Japan

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) frequently show gastrointestinal (GI) toxicity. For instance, gastroduodenal ulcers occur in 20–30% of chronic NSAID users [1–3]. It has also been shown that colonoscopy detects ulcers in the lower GI tract in 3% of chronic NSAID users [4, 5]. Although it had become evident in the 1980s that NSAIDs also damaged the small bowel, in practice, the mucosal injury could not be visualized until capsule endoscopy (CE) and double-balloon endoscopy (DBE) became widely used. While a postmortem examination identified small bowel ulcerations in 21 (8.4%) of 249 NSAID users [3], it has subsequently become evident in CE and DBE studies that NSAIDs cause small bowel mucosal injury more frequently, with a prevalence of up to 70% [6–8].

Recent clinical studies have shown that the incidence of upper GI injury was lower in subjects treated with selective cyclooxygenase (COX)-2 inhibitors than in those treated with non-selective NSAIDs [9–12]. Furthermore, celecoxib, one of the selective COX-2 inhibitors, has been shown to cause small bowel mucosal injury and lower GI events less frequently than non-selective NSAIDs [12–14]. Meloxicam, an agent synthesized as a traditional NSAID, also has a selective inhibitory action against COX-2 [15, 16]. In vitro studies showed that meloxicam had less potent inhibitory action on the synthesis of prostaglandin E, 6-keto-prostaglandin $F_{1\alpha}$, and thromboxane B_2 in human gastric mucosa when compared to indomethacin [17]. Ex vivo analysis of monocytes obtained from meloxicam-pretreated humans revealed that the drug had a five- to tenfold higher inhibitory

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effect on COX-2 than on COX-1 [18–20]. In clinical trials, meloxicam was associated with a lower incidence of upper GI toxic events when compared to other traditional NSAIDs [21–23]. However, small bowel mucosal injury caused by meloxicam has not been examined to date.

In order to examine whether selective COX-2 inhibitors are protective against small bowel injury in humans, and to investigate possible differences between the small bowel toxicity of two selective COX-2 inhibitors, celecoxib and meloxicam, we performed a prospective, double-blind, randomized, controlled study.

Methods

Study design

This study was a prospective, double-blind, randomized trial. Prior to randomization, all subjects underwent laboratory tests (complete blood cell count, serum chemistry, and detection of *Helicobacter pylori* antibody), an electrocardiogram (ECG), and a baseline CE. Any subjects who had abnormal laboratory test results or an abnormal ECG were excluded from the study. Subjects who had small bowel erosions or ulcers at baseline CE were also excluded. All remaining subjects were then randomized, by a computer-generated randomization system, to receive either celecoxib (200 mg twice daily) or meloxicam (10 mg once daily) for 2 weeks. The dose of each drug was determined on the basis of the dose approved by the Japanese Ministry of Health and Welfare and applied to other clinical trials [24, 25]. In both groups, omeprazole (20 mg once daily) was given in consideration of possible gastric mucosal injury. Celecoxib and meloxicam were prepared in dummy capsules and the subjects were instructed to take a capsule twice per day for 2 weeks. The use of other NSAIDs, aspirin, or anti-ulcer drugs was strictly prohibited during the study period. After 2 weeks of medication, the subjects completed a questionnaire about GI symptoms, underwent repeated laboratory tests, and received a second CE.

The study protocol was approved by the institutional review board of the International University of Health and Welfare Fukuoka Sanno Hospital (FS-2-0903-049), and the study was conducted in accordance with the Helsinki Declaration. This trial has been registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) as number UMIN000003871. All subjects provided their written informed consent before entry into the study.

Subjects

Healthy volunteers with normal physical examinations and normal laboratory test results were eligible for the present

investigation. Exclusion criteria were as follows: (1) a history of peptic ulcers, (2) a history of recent (within a month) use of NSAIDs or aspirin, (3) a history of aspirin-induced asthma, (4) allergy to sulfonamide, (5) recent treatment with anti-ulcer drugs, (6) stenosis of the GI tract, (7) a history of adhesion ileus, (8) pregnant or nursing females, and (9) the presence of other disorders regarded as causing the subject's participation in the present study to be inappropriate.

Capsule endoscopy

The baseline and the second CEs were performed using a PillCam SB (Given Imaging, Yokneam, Israel). After an overnight fast for 12 h, each subject was prepared with sensor arrays and a data recorder, and instructed to swallow the capsule with a small amount of water. CE images were recorded for the subsequent 8 h. All the digital video image streams were downloaded to the Given Imaging Reporting and Processing of Images and Data (RAPID) system.

Two observers (M.E. and Y.M.) independently assessed the CE images. Positive CE findings were classified as mucosal bleeding or mucosal injuries. Mucosal injuries were further divided into ulcers and erosions on the basis of the classification reported by Fujimori et al. [26] and Niwa et al. [27] with slight modifications. Mucosal bleeding was defined as the presence of luminal blood in the small intestine. A large mucosal defect with obvious whitish mucous was defined as an ulcer (Fig. 1a), while a small mucosal break surrounded by redness was regarded as an erosion (Fig. 1b). The small intestine was divided equally into the jejunum and the ileum by the small bowel transit time. If the CE findings were different between the two observers, they then discussed the case until a consensus opinion was reached.

Endpoints

The primary endpoint was the incidence of positive CE findings of any type at the second CE.

The secondary endpoints were the incidence of CE findings in the jejunum and in the ileum, the numbers of each CE finding in subjects with positive CE results, GI symptoms, and the presence or absence of anemia. GI symptoms were assessed at the end of the medication period by using a GI symptom rating scale (GSRS) [28]. Anemia was defined as a decrease in the hemoglobin level by more than 2.0 g/dl from the baseline value.

Statistical analysis

The incidence of small bowel mucosal injury after 2 weeks of celecoxib has been shown to range from 6 to

Fig. 1 Examples of mucosal injury observed by capsule endoscopy (CE) (a ulcer, b erosion)
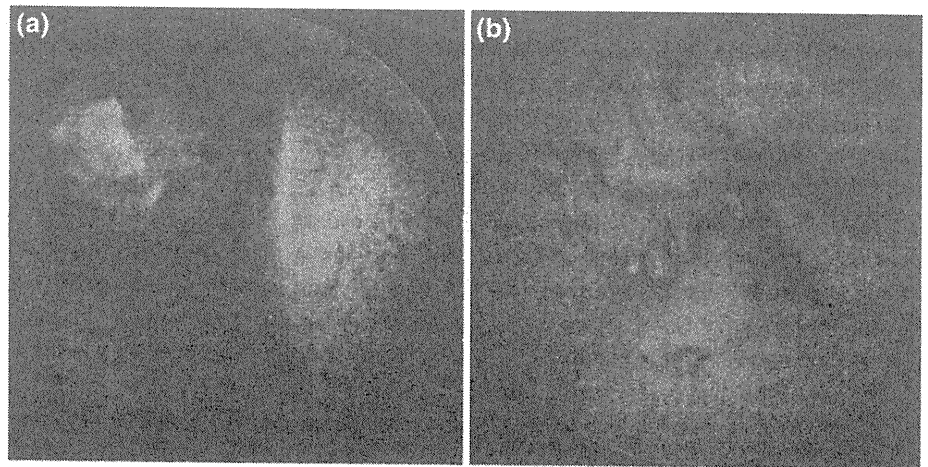
16% [13, 14]. The incidence of small bowel mucosal injury caused by meloxicam was unknown. We thus presumed the incidence to be equivalent to that of non-selective NSAIDs (68–75%) [6, 7]. In the present study, the sample size was calculated on the assumption that the incidence of small bowel mucosal injury would be 10% for celecoxib and 60% for meloxicam. To detect this difference with a 0.05 significance level and a statistical power of 80%, it was calculated that 15 subjects per group would be required.

Parametric data were expressed as medians (ranges). The data were compared between the groups using the Mann–Whitney U-test. Non-parametric data were expressed as frequencies, and analyzed by Fisher's exact probability test or the χ^2 test. A P value of <0.05 was considered to be statistically significant for each test.

Results

Subjects

The study was conducted from April to August 2010. During the study period, 32 subjects were enrolled. A flow chart of the study subjects is shown in Fig. 2. Two subjects were excluded, one because of multiple small bowel ulcers and one because of a slight increase in the serum creatinine level (1.2 mg/dl) at baseline. The remaining thirty subjects were then randomized to either the celecoxib or the meloxicam group. The second CE enabled total enteroscopy in 29 subjects, because the capsule remained in the stomach during the second CE in one subject (who had been taking meloxicam). Consequently, the celecoxib and meloxicam groups comprised 15 subjects and 14 subjects, respectively.

Table 1 shows a comparison of the demographic data in the two groups of study subjects. There were no significant differences in age, gender, or body weight between the two groups. *Helicobacter pylori* infection was detected in 3
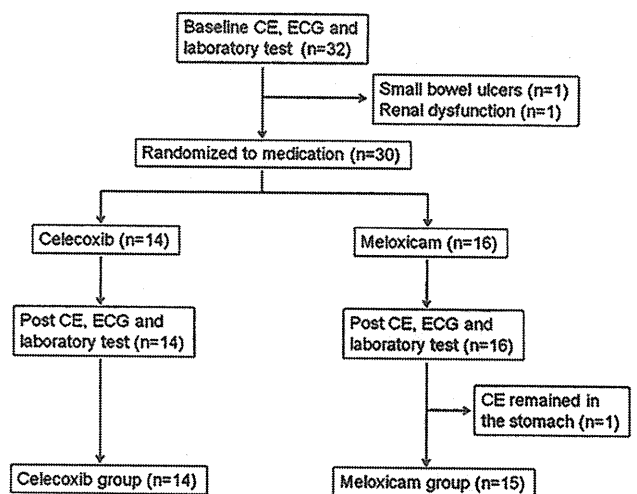

Fig. 2 Flow chart of the study subjects

Table 1 Comparison of demographic data between the celecoxib and meloxicam groups

	Celecoxib group	Meloxicam group	P value
Number of subjects	14	15	
Age (years)	33 (25–50)	30 (24–46)	0.60
Gender (female/male)	6/8	6/9	0.88
Body weight (kg)	66 (45–79)	59 (39–76)	0.68
Helicobacter pylori infection	3	1	0.33
Concurrent medication	1[a]	0	0.48

Parametric data are expressed as medians (ranges)

[a] The subject continued taking an angiotensin II receptor blocker

subjects in the celecoxib group and in one subject in the meloxicam group. The prevalence of the infection was not different between the two groups. One subject in the celecoxib group continued taking concurrent medication for his essential hypertension.

In the subjects who completed the full study protocol, we did not encounter any extra-abdominal symptoms or significant changes in laboratory data.

Capsule endoscopy findings

In each subject, the two observers reported a concordant result as to the presence or absence of positive findings at the second CE. However, there were two subjects in whom the determination of an ulcer or erosion was discordant between the two observers, thereby requiring a discussion. As a result of the discussion, a consensus was reached that there were erosions in 6 subjects in the celecoxib group, three of whom also had ulcers. In the meloxicam group, ulcers were found in 4 subjects, three of whom also had erosions. Consequently, the incidence of small bowel mucosal injuries was not significantly different between the two groups (42.9% in the celecoxib group and 26.7% in the meloxicam group, $P = 0.45$) (Fig. 3). When the total number of mucosal injuries was compared, no significant

difference was found between the celecoxib group (0 [range 0–14]) and the meloxicam group (0 [range 0–18]). Similarly, neither the number of ulcers nor the number of erosions differed between the two groups.

We then compared the severity of mucosal injuries in the two groups in subjects with positive CE findings (Fig. 4). Six subjects in the celecoxib group and four subjects in the meloxicam group were the subjects for the comparison. The number of ulcers in subjects taking celecoxib was 1 (range 0–1), while the number was higher (3 [range 1–3]) in subjects taking meloxicam ($P = 0.02$). The number of erosions was 6 (range 1–13) in subjects taking celecoxib and 13 (range 0–16) in subjects taking meloxicam ($P = 0.52$). The total number of mucosal injuries was no different between the two groups of subjects (6 [range 1–14] in subjects with celecoxib and 16 [range 3–18] in subjects with meloxicam, $P = 0.18$).

Figure 5 shows a comparison of the incidence of jejunal and ileal injuries in the two groups. Ulcers were found only in the ileum, with an incidence of 21% (3 subjects) in the celecoxib group and an incidence of 27% (4 subjects) in the meloxicam group (Fig. 5a). While the incidence of erosions in the jejunum was not different between the two groups (7.1% in the celecoxib group and 6.7% in the meloxicam group, $P = 1.0$), the incidence of ileal erosions was higher in the celecoxib group (42.9%) than in the meloxicam group (20%). However, the difference did not reach statistical significance ($P = 0.25$).

Symptoms, laboratory data, and complications

One subject in the celecoxib group complained of epigastric pain. In the meloxicam group, two subjects experienced abdominal discomfort and one subject had diarrhea. As shown in Table 2, the GSRS score was 17 (range 15–25) in the celecoxib group and 18 (range 15–26) in the meloxicam group. None of the subjects manifested anemia at the end of the medication period.
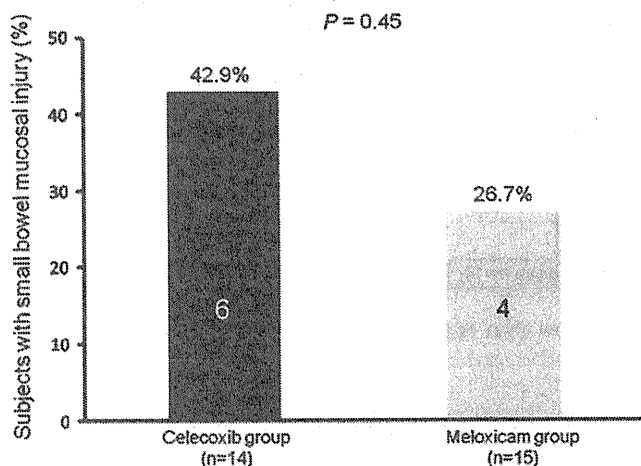

Fig. 3 Comparison of the incidence of small bowel mucosal injury between the celecoxib and meloxicam groups

Fig. 4 Comparison of the number of lesions in subjects with positive CE results (a number of ulcers, b number of erosions)
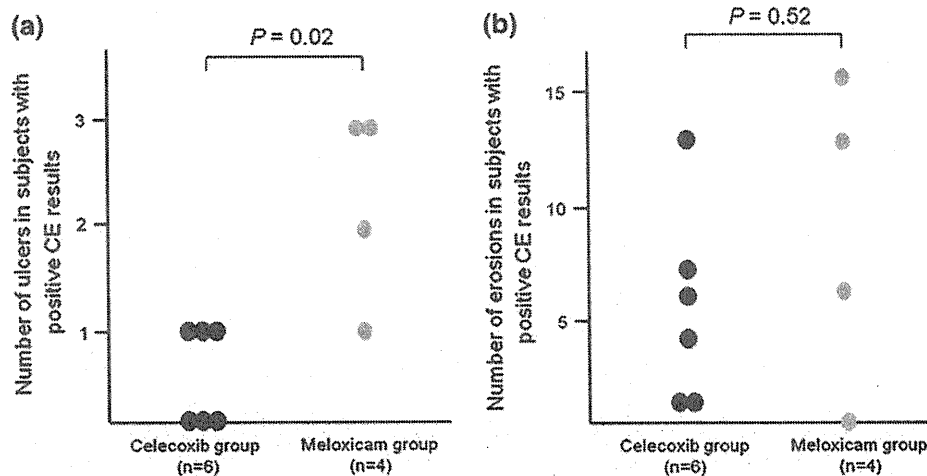
Fig. 5 Comparison of the incidence of small bowel mucosal injuries between the two groups according to their site (a ulcers, b erosions)
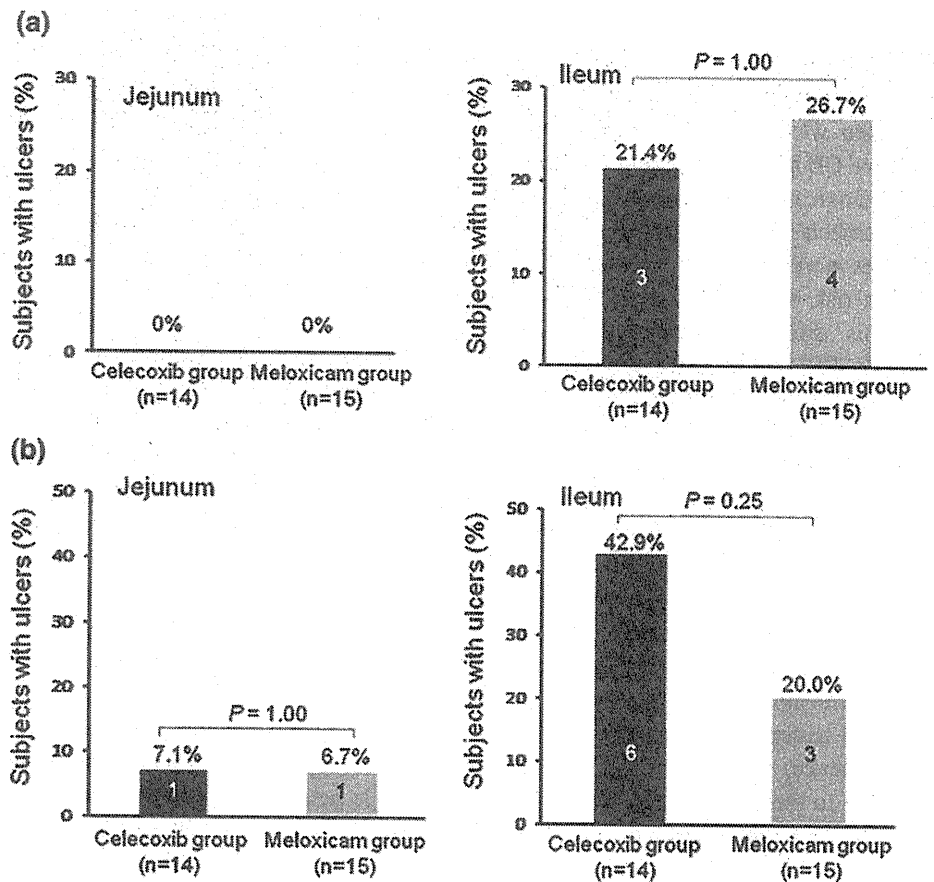

Table 2 Abdominal symptoms and laboratory data

	Celecoxib group ($n = 14$)	Meloxicam group ($n = 15$)	P value
Symptoms	1	3	0.60
GSRS	17 (15–25)	18 (15–26)	0.52
Anemia	0	0	

Anemia was defined as a decrease in the hemoglobin level of at least 2.0 g/dl from baseline

GSRS gastrointestinal symptom rating scale, *GSRS* data are expressed as medians (ranges)

Discussion

NSAIDs and selective COX-2 inhibitors are generally classified by their COX-2/COX-1 selectivity determined by in vitro or ex vivo experiments. In this regard, meloxicam and celecoxib are classified in the same category of NSAIDs, with selectivity ranging from 5 to 50 [29, 30]. However, the relative risk of upper GI toxicity is threefold higher with meloxicam than with celecoxib [31]. Lanas et al. [32] reported a much higher risk of upper GI bleeding in patients administered meloxicam than in those administered celecoxib in a hospital-based, case–control study. These observations suggest that the in vivo COX-2/COX-1

selectivity of each of these NSAIDs is different from their in vitro and ex vivo selectivities, and that the in vitro and ex vivo selectivities are not predictive of GI toxicity. We thus hypothesized that the incidence and the severity of small bowel damage would be different between celecoxib and meloxicam. In accordance with prior clinical trials, we carried out a double-blind prospective study with healthy subjects treated with short-term NSAIDs [13, 14]. As has been confirmed in other prospective studies treating healthy volunteers [7, 13, 14], we found small bowel mucosal lesions in 3% of our subjects prior to the administration of the test drugs.

Our results indicated that the incidence of small bowel mucosal damage induced by celecoxib (43%) was not different from that induced by meloxicam (27%), with rather a higher value for celecoxib than for meloxicam. Interestingly, the incidence of celecoxib-induced small bowel mucosal damage in our subjects was equivalent to that induced by diclofenac or naproxen in Western and Eastern subjects verified by randomized trials [7, 13, 27, 33] and it was higher than that induced by ibuprofen in Western subjects [14]. It thus seems reasonable to conclude that the selective COX-2 inhibitors available at present are not unequivocally safe for the small bowel. However, because celecoxib and meloxicam have anti-COX-1

properties, it is still possible that COX-1 inhibition contributes to the pathogenesis of the mucosal damage even in subjects treated with selective COX-2 inhibitors.

When we compared the CE findings in subjects with positive CE results, we found a greater number of ulcers in the meloxicam-treated subjects than in the celecoxib-treated subjects. This observation suggests that meloxicam induces more severe mucosal lesions in subjects who are at a high risk of NSAID enteropathy. Possible explanations for this difference between meloxicam and celecoxib include differences in the effects of the two drugs on the enterohepatic recirculation [34], in their effects on bacterial flora and bile acid composition, and presumably, in their effects on in vivo COX-2/COX-1 selectivity. Because severe mucosal damage is likely to cause GI complications such as bleeding and perforation, celecoxib may be safer than meloxicam for the small bowel.

In both our celecoxib and meloxicam groups, most mucosal damage was found in the distal part of the small bowel. It has been confirmed that NSAIDs increase intestinal permeability through enterocytic mitochondrial damage and a decrease in prostaglandin synthesis, and, as a consequence, the intestinal mucosa becomes more susceptible to the actions of luminal agents such as bile acid, bacterial flora, and ingested foods [34–37]. Changes in the composition of bile acids and an increase in bacterial flora in the ileum may explain the more severe mucosal damage at this site [37]. A similar trend in the distribution of mucosal injuries has been confirmed in recent studies using other NSAIDs [26, 38, 39], indicating that the ileum seems to be the predominant site prone to mucosal injury in patients taking NSAIDs or COX-2 inhibitors.

The incidence of small bowel mucosal injuries in our celecoxib group was 43%, which was much higher than was predicted (10%). We predicted the incidence of small bowel mucosal injury in the celecoxib group based on the prospective studies done by Goldstein et al. [13, 14], and this discordant result may therefore have been a consequence of the differences in subjects' ethnicities and physiques between the studies done by Goldstein et al. [13, 14] and our present trial. In fact, the body weight of our subjects (median 59 kg) was much lower than that in the study by Goldstein et al. [14] (73 kg). However, it should also be noted that in an observational study done by Maiden et al. [40] in the United Kingdom, CE detected minute small bowel mucosal injuries in 50% of patients taking COX-2 inhibitors (celecoxib, etoricoxib, rofecoxib, or valdecoxib). It thus seems possible that COX-2 plays a significant role in the preservation of the mucosal integrity of the small bowel, and the inhibition of COX-2 can easily lead to mucosal breaks.

Our present study has some limitations. First, because the predicted incidence of mucosal injury in the celecoxib

group was lower than the actual incidence, we should have recruited a larger number of subjects for each group to prove an insignificant difference in the incidence of mucosal injuries between the two groups. We thus cannot deny a significantly higher incidence of injuries in the celecoxib group. However, our conclusion that celecoxib possibly damages the small bowel should not be modified. Second, the small sample size suggests that there may be a type 2 error in the comparison of the severity of mucosal injuries, which means that the number of ulcers was not actually different between the two groups. Finally, subjects in the meloxicam group were administered a 10-mg dose of meloxicam, which is the standard dose in Japan but is lower than that in Western countries (15 mg).

In conclusion, our prospective study indicated that the incidence of small bowel mucosal damage was not different between subjects treated with celecoxib and those treated with meloxicam, suggesting that selective COX-2 inhibitors are not completely safe for the small bowel. Our sub-analysis of subjects with positive CE findings suggested celecoxib to be less harmful than meloxicam, indicating that factors other than in vitro COX-2/COX-1 selectivity may be associated with small bowel toxicity. The conspicuously high incidence of mucosal damage in our subjects treated with celecoxib warrants further studies to establish the role of selective COX-2 inhibitors for the prevention of small bowel injuries in patients scheduled to receive long-term NSAID treatment.

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INTERNATIONAL FORUM

Target biopsy or step biopsy? Optimal surveillance for ulcerative colitis: a Japanese nationwide randomized controlled trial

Toshiaki Watanabe · Yoichi Ajioka · Takayuki Matsumoto · Naoki Tomotsugu · Toru Takebayashi · Eisuke Inoue · Bunei Iizuka · Masahiro Igarashi · Yasushi Iwao · Kazuo Ohtsuka · Shin-ei Kudo · Kiyonori Kobayashi · Miwa Sada · Takayuki Matsumoto · Ichiro Hirata · Kazunari Murakami · Masakazu Nagahori · Kenji Watanabe · Nobuyuki Hida · Fumiaki Ueno · Shinji Tanaka · Mamoru Watanabe · Toshifumi Hibi

Abstract Colorectal cancer is one of the complications of ulcerative colitis (UC) and the risk of cancer increases as the duration of the disease becomes longer. Surveillance colonoscopy has been considered to be important for the early detection and early treatment of colorectal cancer, especially in longstanding UC. Because it is not always easy to detect neoplastic lesions in UC endoscopically, guidelines recommend the use of step biopsy in surveillance, in which either 4 biopsy specimens for every 10 cm

or a total of 33 or more biopsy specimens are obtained. However, it has been pointed out that a step biopsy obtaining several tens of biopsy specimens may not be an ideal method in terms of invasiveness to the patient or medical cost. Instead of step biopsy, recent studies report the usefulness of target biopsy, in which biopsy tissues are obtained only from regions suspected of neoplasia. Therefore, the Research Group for Intractable Inflammatory Bowel Disease of the Ministry of Health, Labour and

T. Watanabe (⊠)
Department of Surgery,
Teikyo University School of Medicine, 2-11-1 Kaga,
Itabashi-ku, Tokyo 173-8605, Japan
e-mail: toshwatanabe@yahoo.co.jp

Y. Ajioka
Niigata University Graduate School of Medical
and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku,
Niigata 951-8510, Japan
e-mail: ajioka@med.niigata-u.ac.jp

T. Matsumoto · N. Hida
Department of Lower Gastroenterology,
Hyogo College of Medicine, 1-1 Mukogawa-cho,
Nishinomiya, Hyogo 663-8501, Japan
e-mail: matsut@hyo-med.ac.jp

N. Hida
e-mail: hidanm@tc4.so-net.ne.jp

N. Tomotsugu
Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: naokit77@gmail.com

T. Takebayashi
Department of Preventive Medicine and Public Health,
School of Medicine, Keio University,
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: ttake@sc.itc.keio.ac.jp

E. Inoue
Kitasao University, 5-9-1 Shirokane, Minato-ku,
Tokyo 108-8641, Japan
e-mail: einoue@kde.biglobe.ne.jp

B. Iizuka
Department of Gastroenterology,
Tokyo Women's Medical University, 8-1 Kawada-cho,
Shinjuku-ku, Tokyo 162-8666, Japan
e-mail: b-izk-kfh@ezweb.ne.jp

M. Igarashi
Cancer Institute Ariake Hospital, 3-8-1 Ariake,
Koto-ku, Tokyo 135-8550, Japan
e-mail: masahiro.igarashi@fcr.or.jp

Y. Iwao
Center for Diagnostic and Therapeutic Endoscopy,
Keio University School of Medicine, 35 Shinanomachi,
Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: yiwao@ac.itc.keio.ac.jp

K. Ohtsuka · S. Kudo
Digestive Disease Center,
Showa University Northern Yokohama Hospital,
35-1 Chigasaki-chuo, Tsuzuki, Yokohama 224-8503, Japan
e-mail: ohts@med.showa-u.ac.jp

S. Kudo
e-mail: kudos@med.showa-u.ac.jp

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