Table 1 Characteristics of enrolled subjects

	Helicobacter pylori negative			*Helicobacter pylori* positive	
	Young	Nonelderly	Elderly	Nonelderly	Elderly
Male/female	8/7	8/8	7/7	9/8	8/8
Age (years)	22.9 ± 1.9	49.9 ± 1.2	75.1 ± 2.1	50.9 ± 1.4	75.1 ± 2.1
Height (cm)	167.3 ± 8.6	166.2 ± 8.0	159.1 ± 6.8	165.5 ± 7.6	159.9 ± 9.5
Body weight (kg)	56.9 ± 8.2	59.8 ± 10.9	61.0 ± 6.4	61.5 ± 12.9	58.5 ± 10.9
BMI (kg/m^2)	20.3 ± 2.0	21.6 ± 3.2	24.1 ± 2.0	22.3 ± 3.3	22.8 ± 2.8
BAO (mEq/h)	2.8 ± 1.4	1.5 ± 1.3	1.3 ± 1.5	1.0 ± 1.5	0.4 ± 0.7
MAO (mEq/h)	18.0 ± 7.8	17.9 ± 4.7	12.6 ± 6.7	12.0 ± 9.0	6.5 ± 5.0
Basal volume (mL/h)	57.7 ± 36.5	24.5 ± 15.4	18.8 ± 10.7	27.4 ± 16.4	20.4 ± 12.6
Maximal volume (mL/h)	157.4 ± 52.9	140.8 ± 35.5	100.6 ± 45.6	127.2 ± 58.6	85.5 ± 43.4
Gastrin (pg/mL)	57.9 ± 20.8	55.2 ± 17.1	88.3 ± 53.8	150.3 ± 124.9	213.9 ± 247.1
Pepsinogen I (ng/mL)	39.9 ± 16.3	45.5 ± 13.9	51.4 ± 30.8	62.2 ± 16.1	54.3 ± 32.8
Pepsinogen II (ng/mL)	5.9 ± 3.1	7.0 ± 2.6	9.9 ± 5.5	23.5 ± 13.1	25.7 ± 12.5
Pepsinogen I/pepsinogen II ratio	7.7 ± 3.4	6.8 ± 1.2	5.1 ± 1.4	3.1 ± 1.2	2.2 ± 1.1

Data are expressed as the mean ± the standard deviation.

BAO basal acid output, *BMI* body mass index, *MAO* maximal acid output

H. pylori-negative subjects (Table 1). The pepsinogen I to pepsinogen II ratio decreased with age, irrespective of *H. pylori* infection. However, even in the elderly without *H. pylori* infection, the mean value of the ratio was 5.1 ± 1.4, which was above the cutoff value of 3 for diagnosing atrophic gastritis.

Correlation of gastric acid secretion with age and BMI

Next, we focused on the correlation between gastric acid secretion and age in *H. pylori*-negative subjects to assess whether gastric acid secretion was affected by age or sex without the influence of that infection. In the young group, BAO in males was higher than in females, and BAO in males gradually decreased with age, which showed a significant correlation among the age groups ($r = -0.578$; data not shown). In contrast, BAO in females was not significantly different among the age groups (data not shown). Likewise, in the *H. pylori*-negative subjects, MAO in males was higher than in females and then gradually decreased with age, with a significant correlation seen among the age groups ($r = -0.638$; Fig. 3a). In contrast, MAO in females was unchanged with age, and the regression coefficient was significantly different from that for males ($p < 0.01$). A similar tendency was seen with *H. pylori*-positive subjects, although the differences were not significant.

Previous findings regarding the relationship between MAO and body size (body weight, body height, BMI) are conflicting [14, 20]. In the present study, although MAO showed a modestly positive correlation with body weight and body height irrespective of *H. pylori* infection (data not shown), BMI was correlated with MAO in subjects with *H. pylori* infection, but not in subjects without *H. pylori* infection (Fig. 3b).

Comparisons with previous findings from the 1970s and the 1990s

Finally, to evaluate whether gastric acid secretion in Japanese individuals has changed over the past two to four decades, we compared the present findings with those previously reported by our group [16]. BAO is likely to be susceptible to influences from stress and various other factors [21], and the values for MAO in each period were compared. MAO in the present nonelderly subjects without *H. pylori* infection was increased as compared with that in the 1970s ($p < 0.025$), but there were no significant differences in nonelderly and elderly subjects without *H. pylori* infection when compared with MAO in the 1990s (Fig. 4a). On the other hand, MAO in elderly subjects with *H. pylori* infection was significantly lower in the present study than in the 1990s ($p < 0.01$; Fig. 4b). Furthermore, serum gastrin concentration in the present nonelderly and elderly *H. pylori*-negative subjects was significantly lower than that in such subjects determined in the 1990s ($p < 0.005$ and $p < 0.025$, respectively; Fig. 5a), whereas that concentration in the present *H. pylori*-positive subjects was not different from the measurement in the 1990s (Fig. 5b). Together, these findings indicate that MAO has not increased over the past two decades in Japanese individuals irrespective of the presence of *H. pylori* infection.

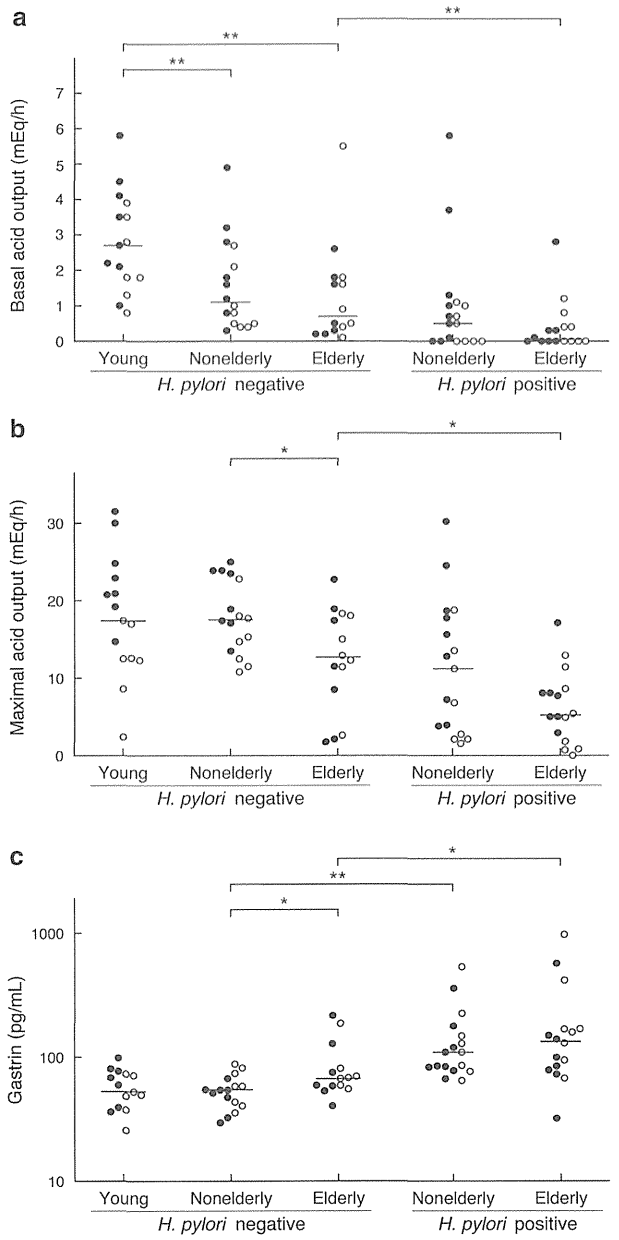


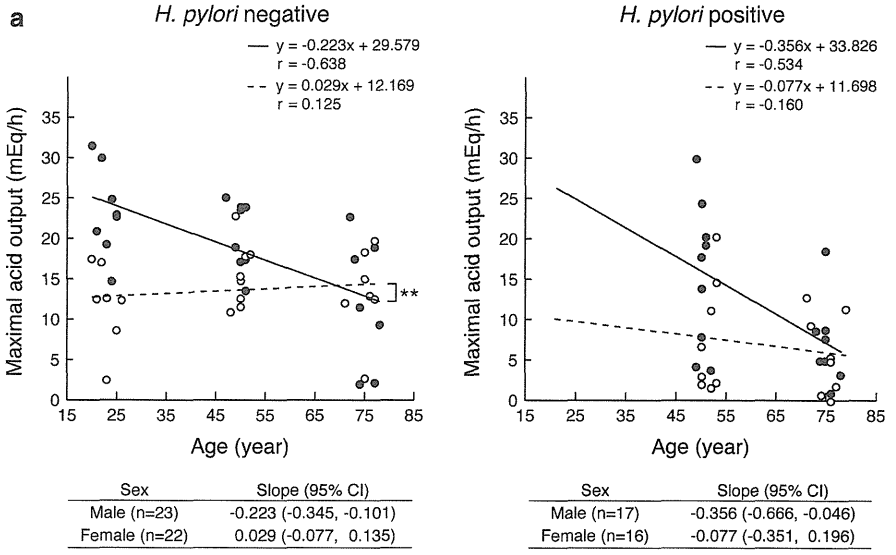
Fig. 2 Effects of age and *Helicobacter pylori* infection on basal acid output (**a**), maximal acid output (**b**), and serum gastrin concentration (**c**). Each data point represents an individual subject. The median is shown by a *horizontal bar*. *Black circles* males, *white circles* females, *one asterisk* $p < 0.05$, *two asterisks* $p < 0.01$

Discussion

In Western countries, the level of gastric acid secretion has been reported to remain the same or increase with age [14, 22, 23]. In contrast, investigations targeting Japanese subjects have shown that gastric acid secretion is decreased in elderly individuals owing to encroaching atrophic gastritis [24, 25]. The reason for this difference between Western and Japanese populations remains unknown, although it has been speculated that the higher prevalence of *H. pylori* infection in Japan may be associated with a decrease in gastric acid secretion with age [26, 27]. On the other hand, Kinoshita et al. [16] reported that gastric acid secretion was lower in elderly subjects than in nonelderly subjects without *H. pylori* infection, which suggested the presence of factors other than that infection. In addition, another Japanese group found that advancing age had no influence on gastric acid secretion in *H. pylori*-negative subjects [15], although only a single subject older than 70 years and without *H. pylori* infection was included in that study. Furthermore, these studies were conducted more than 10 years ago, and the recent trend of gastric acid secretion in Japan has not been assessed.

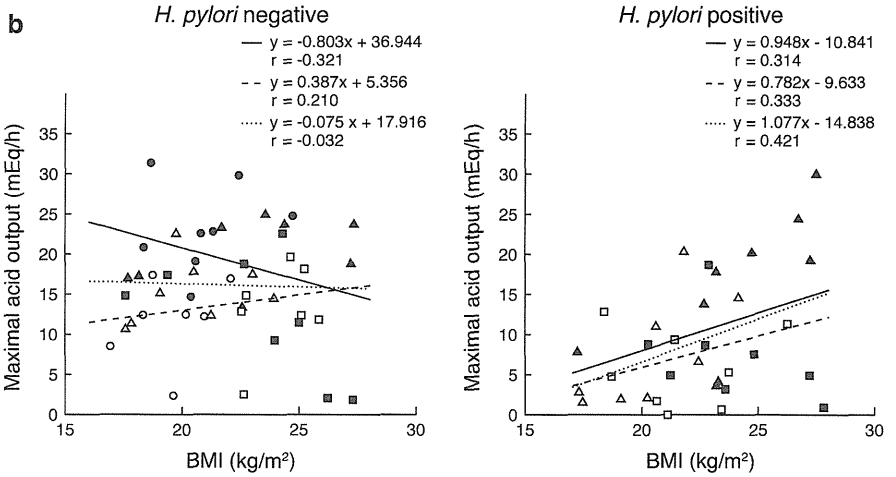
As noted already, the effects of age in individuals without *H. pylori* infection remain largely unknown, likely owing to inconsistency among past study protocols, such as the age distribution, the gastric acid secretion measurement method, and the methods used for evaluation of *H. pylori* infection. To assess the effects of aging more precisely, we confirmed the presence of infection in each subject by using both urea breath testing and serum IgG antibody testing to exclude serologically negative *H. pylori* infections. We also analyzed younger subjects (young group) to assess the effect of age on gastric acid secretion. Our findings strongly support the concept that gastric acid secretion decreases with age in individuals without *H. pylori* infection, especially in those older than 70 years.

An interesting finding of our study is that MAO remained unchanged with age in females, whereas it decreased with age in males, despite the fact that the serum pepsinogen I to serum pepsinogen II ratio decreased with age regardless of sex. Although the effects of age on gastric acid secretion have yet to be clearly defined, those changes may differ between males and females. Animal studies have suggested that female sex hormone might have an effect on gastric acid secretion [28]. In ovariectomized rats, the parietal cell mass as well as basal acid secretion increases, suggesting that estrogen could regulate gastric acid production. Likewise, several studies have shown that male sex hormones are important for regulating gastric acid secretion. In contrast to ovariectomy, orchidectomy of male rats decreases both the number of parietal cells in the gastric mucosa and basal acid secretion [28]. Another animal study also has shown that an orchidectomy resulted in a significant decrease in acid output as compared with the acid output in sham-operated rats, despite the finding that plasma gastrin concentration was significantly increased in that group. In addition, administration of testosterone to animals that underwent an orchidectomy resulted in a significant increase in acid output to nearly the same value as shown in a vehicle control [29, 30]. The



Sex	Slope (95% CI)
Male (n=23)	-0.223 (-0.345, -0.101)
Female (n=22)	0.029 (-0.077, 0.135)

Sex	Slope (95% CI)
Male (n=17)	-0.356 (-0.666, -0.046)
Female (n=16)	-0.077 (-0.351, 0.196)



Sex	Slope (95% CI)
Male (n=23)	-0.8029 (-1.8761, 0.2703)
Female (n=22)	0.3868 (-0.4528, 1.2265)
Total (n=45)	-0.0749 (-0.8038, 0.6540)

Sex	Slope (95% CI)
Male (n=17)	0.9478 (-0.6280, 2.5236)
Female (n=16)	0.7821 (-0.4891, 2.0533)
Total (n=33)	1.0765 (0.2278, 1.9253)

Fig. 3 Correlation between maximal acid output and age (**a**) and between maximal acid output and body mass index (*BMI*; (**b**) divided by sex. *Left Helicobacter pylori*-negative subjects, *right H. pylori*-positive subjects. Each data point represents an individual subject. *Black symbols* males, *white symbols* females, *solid line* males, *dashed line* females, *dotted line* total. For **b**, *circles*, *triangles*, and *squares* indicate young, nonelderly, and elderly, respectively. *CI* confidence interval, *two asterisks* $p < 0.01$

relationship between sex hormone and gastric acid remains conflicting [31], and at least in part, the gender effect may be due to differences in parietal cell mass or function between males and females, regulated by sex hormone. Assessments of human subjects are lacking, although the results of those previous animal experiments are consistent with the present findings showing that MAO in nonelderly males was higher than in females and only male subjects showed decreased MAO with age. Moreover, this sex difference in gastric acid secretion in association with age may partly explain the female predominant GERD prevalence in Japanese elderly subjects. Additional examinations are needed to evaluate this relationship in detail [32–35]. Nonetheless, the grade of gastric atrophy as assessed by the pepsinogen I to pepsinogen II ratio were within the normal range (above 3) even in elderly without *H. pylori* infection. Therefore, our findings may not affect the interpretation of the pepsinogen I to pepsinogen II ratio as a screening tool for gastric cancer in Japan.

When gastric acid secretion in Japanese was compared between the 1990s and the 1970s, it was clearly augmented in the later period, regardless of *H. pylori* infection [16]. This increase over a few decades has been speculated to be related to changes in not only dietary habits but also lifestyle. From the 1960s onward, drastic socioeconomic growth and the Westernization of lifestyle, such as increased consumption of a high-fat diet and a higher percentage of individuals performing sedentary work, have spread throughout Japan. According to a national health and nutrition examination survey, there has been a

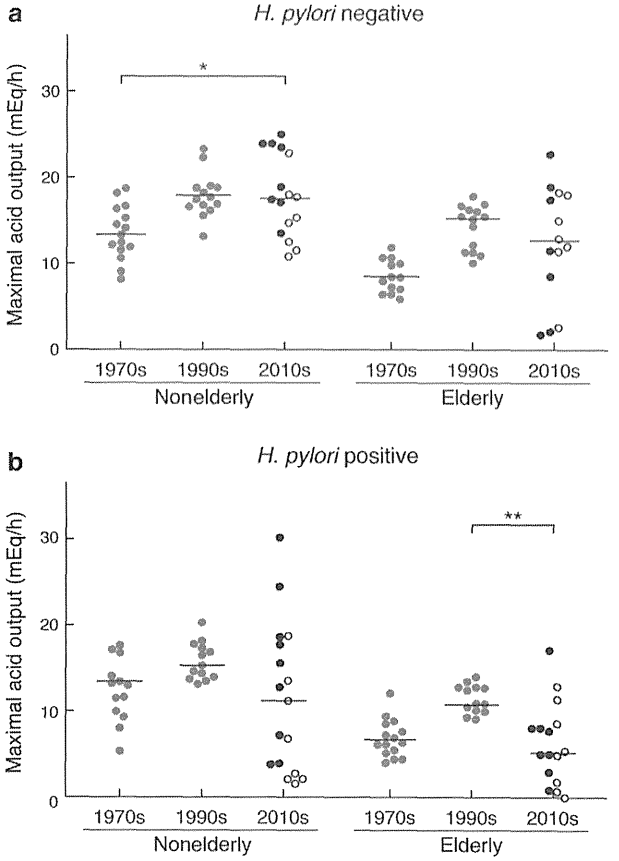


Fig. 4 Comparison of gastric acid secretion in present subjects with data obtained in the 1970s and the 1990s. The subjects were divided by *Helicobacter pylori*-negative status (**a**) and *H. pylori*-positive status (**b**). Each data point represents an individual subject. The median is shown by a *horizontal bar*. *Black circles* males, *white circles* females, *gray circles* data from the previous study by Kinoshita et al. [16], *one asterisk* $p < 0.025$, *two asterisks* $p < 0.005$

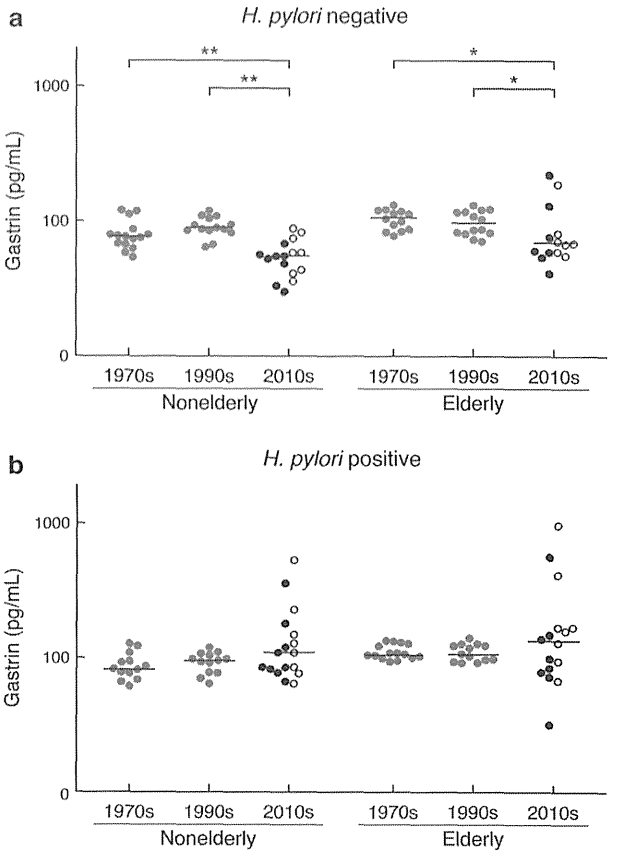


Fig. 5 Comparison of serum gastrin concentration in present subjects with data obtained in the 1970s and the 1990s. The subjects were divided by *Helicobacter pylori*-negative status (**a**) and *H. pylori*-positive status (**b**). Each data point represents an individual subject. The median is shown by a *horizontal bar*. *Black circles* males, *white circles* females, *gray circles* data from the previous study by Kinoshita et al. [16], *one asterisk* $p < 0.025$, *two asterisks* $p < 0.005$

remarkable increase in dietary fat intake and a modest increase in protein consumption during this period. However, fat intake and protein consumption in Japanese have not increased from the 1990s to the present. Stress is thought to be one of the factors related to increased gastric acid secretion via gastrin secretion [21, 36]. Our data showing that serum gastrin concentration has significantly decreased from the 1990s to the present suggest that physiological and/or mental stress factors may change in association with a more matured society. As a result, gastric acid secretion may not have increased over the past two decades. In contrast, MAO in elderly subjects with *H. pylori* infection was significantly lower in the present than in the 1990s, whereas serum gastrin concentration did not differ between the groups. Although the precise mechanism remains unclear, several possibilities could be considered. In subjects with *H. pylori* infection, gastric acid secretion mainly depends on the degree of gastric mucosal atrophy, which is associated with the decrease in acid secretary capacity. Therefore, a larger number of subjects with advanced gastric atrophy may be included in the present study. Another possibility was the influence of the concurrent environmental factor, including lifestyle (dietary habit, salt intake, and smoking). Nonetheless, this group should be carefully followed up because advanced-stage gastric mucosal atrophy is a risk factor for developing gastric cancer.

MAO was associated with BMI in subjects with *H. pylori* infection, but not in subjects without *H. pylori* infection, suggesting that body size has a slight influence on gastric acid secretion in subjects without *H. pylori* infection. Our findings indicated that decreased gastric acid secretion resulting from progression of the gastric atrophy caused by *H. pylori* infection may be associated with nutritional status. Consistently, the prevalence of *H. pylori* colonization has been shown to be inversely related to the prevalence of obesity [37]. In addition, previous controlled trials showed that after successful *H. pylori* eradication

patients experience a significant increase in weight that was not observed in control subjects who received placebo instead of *H. pylori* eradication [38]. Other factors linked to *H. pylori* rather than *H. pylori* infection itself may be causal for the observed association between *H. pylori* and obesity. Therefore, investigation of the effects of visceral fat and adipocytokines, including ghrelin and leptin, and the microbiome on gastric acid secretion is needed to reveal this relationship [39, 40]. Nevertheless, gastric acid secretion in Japanese individuals still seems to be lower than that in Europeans and North Americans [14], suggesting that race or genetic factors are as important as lifestyle factors.

The present study has some limitations. First, the number of subjects in each group was lower than that in previous studies conducted in Western countries [22, 23]. However, it is important to emphasize that a major aim of this study was to compare current with previous data obtained in the 1990s. To establish the same conditions as in our previous study, the enrolled subjects were consistently assigned to each group. Second, the measurement methods used for BAO and MAO were slightly different from those of the previous study, as phenolphthalein was used for neutralization titration in the previous study and a pH meter was used in the present protocol. Nevertheless, we calculated the deviation of acidity using the previous method and adjusted the results appropriately. As a result, we consider that the present study is more reliable than other studies to compare the data obtained with data obtained in the 1990s.

In conclusion, in contrast to the increased prevalence of GERD, gastric acid secretion has not increased over the past two decades in Japanese individuals. In subjects without *H. pylori* infection, gastric acid secretion decreased with age in males but not in females, which may partly explain the sex differences seen in the prevalence of age-related GERD.

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Conflict of interest Hiroto Miwa has received research grants from AstraZeneca and Daiichi-Sankyo, Kazuhide Higuchi has received research grants from Daiichi-Sankyo, and Yoshikazu Kinoshita has received research grants from AstraZeneca and Daiichi-Sankyo. Hiroto Miwa has served in speaking and teaching roles for AstraZeneca and Daiichi-Sankyo, Kazuhide Higuchi has served in speaking and teaching roles for Daiichi-Sankyo, and Yoshikazu Kinoshita has served in speaking and teaching roles for AstraZeneka and Daiichi-Sankyo. Norihisa Ishimura, Yasuko Owada, Masahito Aimi, Tadayuki Oshima, Tomoari Kamada, Kazuhiko Inoue, Hironobu Mikami, and Toshihisa Takeuchi declare that they have no conflict of interest.

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The Number and Distribution of Eosinophils in the Adult Human Gastrointestinal Tract

A Study and Comparison of Racial and Environmental Factors

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Abstract: There are surprisingly limited data regarding normal counts or distribution of eosinophils in the gastrointestinal tract, despite the increasing incidence of eosinophilic gastrointestinal tract diseases. Moreover, there are no published reports on the eosinophil number throughout the gastrointestinal tract of adults or Asian populations, or those investigating the effect of race on eosinophil count. First, in our study, the number of eosinophils from each portion of the gastrointestinal mucosa was quantified on biopsy slides from a Japanese adult population (132 samples). Next, the surgical resections from Japanese (110 samples), Japanese Americans (64), and Caucasians (57) were used to investigate the racial and environmental effects. Our results with the Japanese biopsy samples showed a significant increase in the number of eosinophils from the esophagus to the right colon (mean ± SD/mm^2: 0.07 ± 0.43 for the esophagus, 12.18 ± 11.39 for the stomach, and 36.59 ± 15.50 for the right colon), compared with a decrease in the left colon (8.53 ± 7.83). Investigation using surgical samples showed that the distribution patterns in the gastrointestinal tract were very similar among the 3 ethnic groups, and there were no significant differences in the number of eosinophils among these groups, except in the esophageal epithelium. This study is the first report on the normal numbers and distribution of eosinophils throughout the gastrointestinal tract not only of an Asian population but also of adults. Our data suggest that a cutoff value for eosinophil counts, when rendering a diagnosis of eosinophilic gastrointestinal tract disease, should be individualized to the different biopsy sites. Interestingly, race and environmental factors did not seem to have a significant effect on eosinophil densities and distributions.

Key Words: normal, eosinophil, gastrointestinal tract, race

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The number of patients with allergic diseases such as asthma and atopic dermatitis has been increasing with improvement of environmental hygiene and sanitation. Along with this increase, patients suffering from digestive diseases associated with significant eosinophilic infiltration (ie, eosinophilic esophagitis and eosinophilic gastroenteritis) have also been reported to be increasing in number.[1,2] To render a correct diagnosis in these patients, it is important to discern whether eosinophils are pathologically infiltrating the gastrointestinal tract mucosa. However, there are relatively little published data (5 reports[3–7]) in reference to normal eosinophil counts or normal distribution of eosinophils in the gastrointestinal tract, and 3 of these 5 reports were focused only on children in Western countries. No report has investigated the normal distribution of eosinophils from the esophagus to the rectum of adults. Thus, to definitively make a pathologic diagnosis of eosinophilic gastrointestinal tract disease of adults in Asia, it is essential to quantify and characterize the eosinophil content in the normal gastrointestinal tract mucosa of the Asian populace.

Our main purpose was to know the content and distribution of eosinophils throughout the normal gastrointestinal tract mucosa of Japanese (J) using adult biopsy specimens. In addition, to our knowledge, no studies have been published regarding the effect of race on the eosinophil content in the normal digestive tract mucosa. Thus, our second purpose was to compare the number and distribution of eosinophils in the Japanese population with those in Japanese Americans (JA) and Caucasians (W), using surgical specimens.

MATERIALS AND METHODS

Our study was conducted at Shimane University Hospital and the Queens Medical Center. The former is located in Izumo, Japan (latitude 35.37, longitude −227.25), whereas the latter is in Honolulu, Hawaii (latitude 21.31, longitude −157.86). The research was approved by the ethical committees of Shimane University Hospital (approved #: 1145) and Queens Medical Center (approved #: RA 2012 07).

Patients and Sample Collection

The databases of both institutes were searched to identify samples suitable for the present study. The Japanese patients were living in Shimane prefecture located in the western part of Japan, and the American patients were in the state of Hawaii. Normal mucosal biopsy samples of Japanese subjects were taken from gastrointestinal tract mucosa that was endoscopically normal and histologically showed neither neutrophilic nor significant lymphoplasmacytic infiltration. All were outpatients of Shimane University Hospital. Most came to the hospital for their annual medical checkup without prominent symptoms. As is customary in Japan, gastrointestinal biopsies were sometimes taken to histologically confirm the patients' normal condition. Some had diarrhea and were suspected to have microscopic colitis without significant endoscopic findings. None of the patients had allergic diseases and eosinophilia in the peripheral blood, nor were they taking any steroids, antiallergic, or immunosuppressive drugs. The database also showed no past history of allergic diseases. We were able to include 34 esophageal (age 49 to 88, M:F-10:8), 35 gastric (age 25 to 90, M:F-16:15), 23 duodenal (age 37 to 88, M:F-9:14), 15 ileal (age 23 to 72, M:F-5:6), and 25 large intestinal (age 23 to 74, M:F-4:7) biopsy samples in our study. The surgical samples from all 3 ethnic groups were taken from the surgical margins of esophago-gastrointestinal resections for carcinomas of various stages (Table 1). The sampled sites were ≥ 5 cm away from the cancers. These sites macroscopically appeared normal and histologically exhibited no significant inflammation or neoplastic lesions. In addition, the patients who underwent surgery neither had allergic disease nor took any of the medications described above. They also had no eosinophilia in the peripheral blood and had no past history of allergic diseases. The gastric mucosa from both biopsies and surgical resections was free of *Helicobacter pylori* organisms. The period of study was from 1997 to 2012 for the Japanese group and from 2002 to 2012 for the Japanese American and Caucasian groups in Hawaii. Over 2000 gastrointestinal biopsies are taken and submitted to the Department of Pathology at Shimane University Hospital annually, and around 300 gastrointestinal surgeries for cancers are performed a year both at Shimane University Hospital and The Queens Medical Center. Despite the large numbers of biopsies and surgeries, it was very difficult to collect samples suitable for the present study, because we had to exclude so many cases in which the mucosa appeared endoscopically or macroscopically normal. The samples taken from apparently normal mucosa often showed significant lymphoplasmacytic infiltration, especially in surgical cases. This difficulty in sample collection accounts for small numbers and sex differences in the 3 groups.

Eosinophil Count

In this study, we microscopically examined normal gastrointestinal tract mucosa, including esophagus, stomach, and small and large intestines on hematoxylin and eosin–stained slides. Microscopes used were Olympus BX50 for Japanese samples and Olympus BH2 for Japanese American and Caucasian samples. A high-power field (HPF) included an area of 0.237 mm^2 with Olympus BX50 and 0.196 mm^2 with Olympus BH2.

First, we attempted to clarify the numbers and distribution of eosinophils in each portion of the gastrointestinal tract using Japanese biopsy samples. Second, the comparison among the 3 ethnic groups was performed using surgical specimens, because normal biopsy samples from Japanese American and Caucasian groups were not readily available in Hawaii. Almost all of the esophageal biopsy specimens were composed only of stratified squamous epithelium, so that we were unable to evaluate the eosinophil numbers in the lamina propria adequately. However, an eosinophil count could be performed in the epithelium and the lamina propria separately and sufficiently when the surgical specimens were examined. Only the interfoveolar or intercryptal spaces of the mucosa were examined in the stomach and intestines. The areas around lymphoid follicles were excluded. Our cases showed no eosinophilic infiltration into the foveolar or cryptal epithelium. The average number of infiltrating eosinophils per HPF was calculated after counting them in 5 and 10 randomly selected HPFs for the biopsies and surgical specimens, respectively. The density of eosinophils was reported in the number of eosinophils/mm^2.

Statistical Analysis

Because the data were all nonparametric, the Mann-Whitney test was carried out for statistical analysis using SPSS software (version 17, Chicago, IL). A P value < 0.05 was considered to be statistically significant. The Bonferroni correction was used for comparisons among the 3 ethnic groups.

RESULTS

The representative histologic features of biopsy specimens taken from each segment of the gastrointestinal tract are shown in Figure 1. The eosinophil density varied greatly depending on the fields selected even within 1 biopsy slide, a finding consistent with that of Odze et al.[8]

Number and Distribution of Eosinophils in Biopsy Samples From Japanese Patients

Table 2 shows the statistical data obtained from examination of biopsies from each portion of the gastrointestinal tract of Japanese patients. As the sample number was relatively small, the gastric biopsies were not classified into fundus, body, or antrum, neither were the duodenal biopsies into the first through the fourth portions. Regarding the ileal mucosa, samples from only the terminal portion were available. The large intestine is often divided into right and left side anatomically; however, in a retrospective study like this report, it was not possible to clearly discern the exact location of the biopsy site of the transverse colon samples. For convenience, we divided the large intestine into 2 groups: cecum (C), ascending colon (A), and transverse colon (T) as right group (C/A/T) and descending colon (D), sigmoid

TABLE 1. Japanese (J), Japanese American (JA), and Caucasian (W) Samples (Surgical Samples)

	J			JA			W		
	Age (Range [Mean])	Sex	N	Age (Range [mean])	Sex	N	Age (Range [mean])	Sex	N
Esophagus	49-87 (70.5)	M:F = 29:4	33	53-89 (70.2)	M:F = 13:3	16	47-86 (67.4)	M:F = 8:2	10
Stomach	49-85 (70.5)	M:F = 11:5	16	34-93 (71.8)	M:F = 6:9	15	52-89 (68.5)	M:F = 11:1	10
Ileum (terminal)	55-93 (78.9)	M:F = 6:11	17	40-95 (72.2)	M:F = 6:13	19	46-87 (67.5)	M:F = 14:6	20
Large intestine (C/A/T)	55-97 (76.1)	M:F = 2:12	14	44-94 (70.2)	M:F = 2:4	6	45-79 (64.0)	M:F = 4:2	6
Large intestine (D/S/R)	40-90 (72.4)	M:F = 15:15	30	38-86 (68.1)	M:F = 5:3	8	48-90 (66.2)	M:F = 6:5	11

colon (S), and rectum (R) as left group (D/S/R). The mean eosinophil densities ± SD of the esophagus, stomach, duodenum, terminal ileum, C/A/T, and D/S/R were 0.07 ± 0.43/mm^2 (range 0.00 to 2.52/mm^2), 12.18 ± 11.39/mm^2 (range 0.00 to 39.64/mm^2), 33.51 ± 12.88/mm^2 (range 12.64 to 56.44/mm^2), 42.18 ± 35.28/mm^2 (range 10.95 to 119.63/mm^2), 36.59 ± 15.50/mm^2 (range 16.85 to 56.45/mm^2), and 8.53 ± 7.83/mm^2 (range 0.00 to 24.43/mm^2), respectively. Figure 2 is the Box-and-Whisker plot to clearly show the data of Table 2. It reveals a statistically significant increase in the number of eosinophils from the esophagus to the duodenum. There were no significant differences in eosinophil counts among the duodenum, terminal ileum, and the C/A/T of the large intestine, but the number was significantly decreased in the D/S/R of the large intestine. The eosinophil counts varied considerably depending on the segment of gastrointestinal tract examined.

Effect of Race and Environmental Factors

Surgical gastrointestinal tract specimens were collected from Japanese, Japanese American, and Caucasian patients as described above. Because endoscopically normal parts of the gastrointestinal tract are rarely taken for histologic assessment at the Queens Medical Center, it was difficult to draw normal biopsy samples from their pathology database. Therefore, international ethnic comparisons were performed using surgical specimens including esophageal epithelium, esophageal lamina propria, gastric mucosa, terminal ileal mucosa, and C/A/T and D/S/R mucosa of the large intestine. Table 3 shows the data of the international comparisons. The paired data revealed that the number of eosinophils in the intraepithelial layer of the esophagus of Japanese patients was significantly higher than those of Japanese American (JA), Caucasian patients (W), and JA + W patients, when the Mann-Whitney test was used, although the difference between J and W became marginally significant after the Bonferroni correction ($P = 0.064$). There were no significant differences in other segments of the gastrointestinal tract among the 3 ethnic groups. Figure 3 shows the distribution patterns of eosinophils of all 3 ethnic groups with the statistical results. They were quite similar to one another with the terminal ileum and/or C/A/T of the large intestine showing the highest concentration of eosinophils for all 3 groups. The eosinophil content varied significantly depending on the site examined, just as was seen in the Japanese biopsy samples.

DISCUSSION

Eosinophil-associated gastrointestinal tract diseases including eosinophilic esophagitis and eosinophilic gastroenteritis have been prevalent mainly in Western countries.[1,2,9,10] These eosinophilic gastrointestinal tract diseases, along with other allergic diseases such as atopic dermatitis and asthma, have also been on the rise in Asian countries.[11–14] Moreover, the number of adult patients has recently been increasing.[15,16] Pathologic examination plays an important role in making a definitive diagnosis of eosinophilic esophagitis and eosinophilic gastroenteritis, and pathologists must take into account the normal number and distribution of eosinophils in gastrointestinal tract mucosa, when examining histologic specimens from patients with suspected eosinophilic gastrointestinal tract diseases. In addition, pathologists occasionally encounter biopsy specimens from the gastrointestinal tract with seemingly increased number of eosinophils without any other clinical indications of eosinophilic gastrointestinal tract diseases. However, in spite of the importance of pathologic examination, there is only limited information regarding the normal eosinophil count or normal distribution of eosinophils in gastrointestinal tract mucosa,[3–7] rendering morphologic diagnosis of eosinophilic gastrointestinal tract diseases quite subjective. Lowichik and Weinberg[3] and DeBrosse et al[4] investigated the number of eosinophils throughout the gastrointestinal tract, but their reports were focused only on pediatric cases. Saad[6] reported only on pediatric colon cases. Polydorides et al[5] and Lwin et al[7] examined the normal biopsy samples from adults, but their cases were colon and gastric biopsies, respectively. No report has investigated the number of eosinophils throughout the gastrointestinal tract of adults. Furthermore, the studies described above were all from Western countries. There has been no report on the number and distribution of the gastrointestinal tract of an Asian population. To diagnose patients as having eosinophilic esophagitis or eosinophilic gastroenteritis conclusively in Asian countries, it is imperative to know the eosinophil content and distribution in the normal gastrointestinal tract mucosa of Asians. Furthermore, no data have been published in reference to the effect of race and environmental factors on the eosinophil content in the normal gastrointestinal tract mucosa.

In the present study, we first evaluated the content and distribution of eosinophils using Japanese biopsy samples. When the ranges of the average number of each portion of gastrointestinal tract were calculated as the

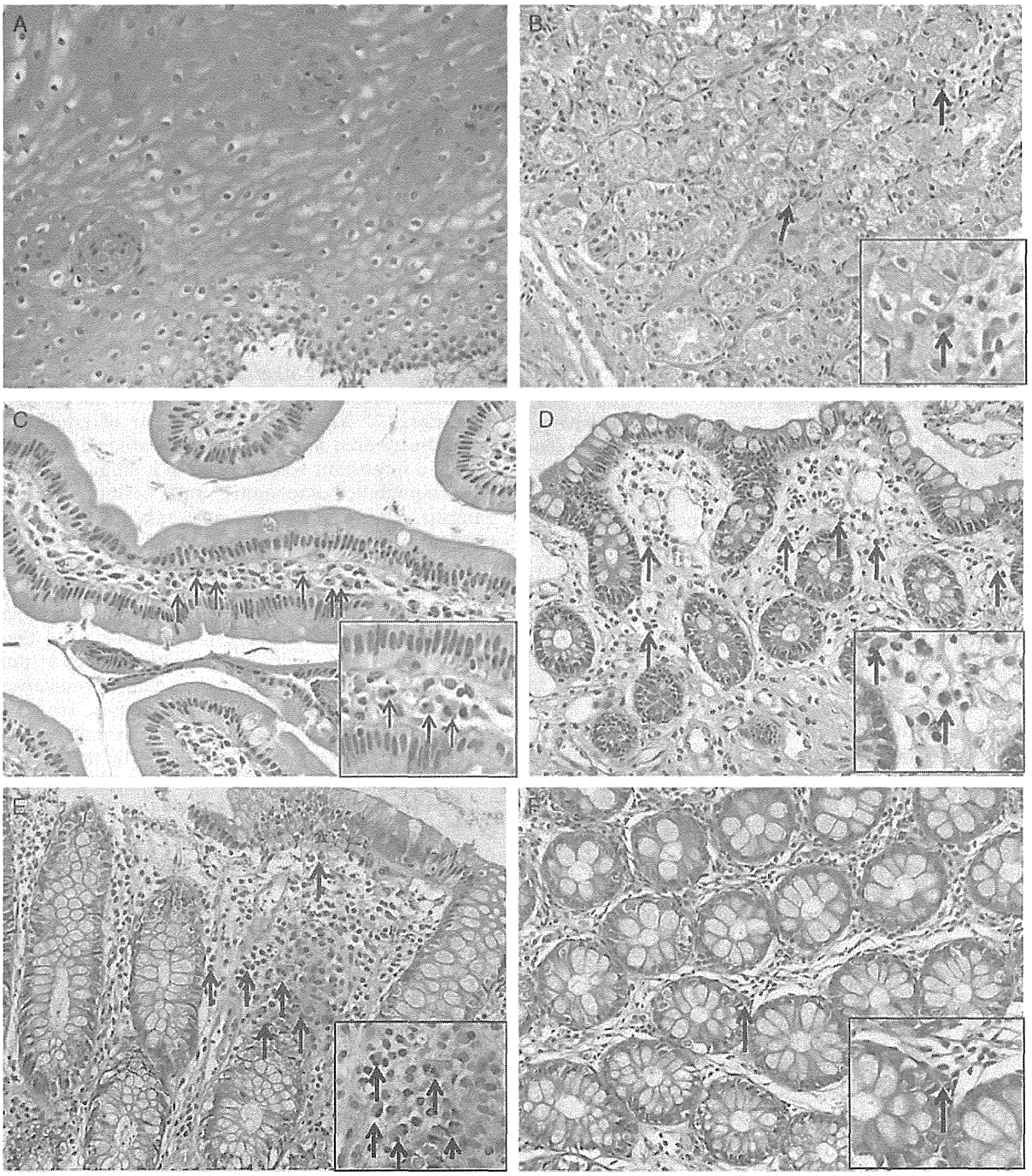

FIGURE 1. Representative histologic features of biopsy samples (hematoxylin and eosin). A, Esophageal epithelium. B, Gastric mucosa. C, Duodenal mucosa. D, Ileal mucosa. E, Ascending colon mucosa. F, Rectal mucosa. The arrows indicate some of the eosinophils present in the lamina propria. Insets of B through F are high-power images clearly illustrating eosinophils.

number/HPF with Olympus BX50, they were 0.00 to 0.60 for the esophagus, 0.00 to 9.39 for the stomach, 3.00 to 13.40 for the duodenum, 2.60 to 28.40 for the terminal ileum, 3.99 to 13.40 for the C/A/T of the large intestine, and 0.00 to 5.80 for the D/S/R. As expected, the number of eosinophils detected in the esophageal biopsy specimens was very low, although we could not adequately assess the subepithelial layer with these suboptimal biopsy samples. The highest detected eosinophil density (2.52/mm^2) could be correlated with an eosinophil

TABLE 2. Eosinophil Count in Japanese Biopsy Samples (Number/mm^2)

	Mean	Median	SD	Variance	Range
Esophagus	0.07	0.00	0.43	0.19	0.00-2.52
Stomach	12.18	7.58	11.39	129.3	0.00-39.60
Duodenum	33.51	35.38	12.83	164.66	12.64-56.44
Ileum (terminal)	42.18	29.49	35.28	1244.63	10.95-119.63
Large intestine (C/A/T)	36.59	43.81	15.50	240.33	16.85-56.45
Large intestine (D/S/R)	8.53	6.32	7.83	61.33	0.00-24.43

count of 0.60/HPF with the use of Olympus BX50. It was accordingly confirmed that virtually no eosinophils were present in the normal esophageal epithelium, a finding in line with what we see in our daily pathology practice. The other highest average eosinophil densities of each segment correlated to 9.39/HPF, 13.38/HPF, 28.35/HPF, 13.38/HPF, and 5.79/HPF, respectively. The number of eosinophils significantly increased from the esophagus to the duodenum but it decreased in the D/S/R of the large intestine. This pattern of distribution is similar to the pediatric cases reported previously.[3,4] However, Saad[6] reported a gradual decrease in the number of eosinophils in the lamina propria from the cecum to the descending colon with another peak in the rectosigmoid. The cause of the difference in distribution pattern between our study and Saad's is unknown. As for adult normal colon, Polydorides et al[5] reported that lamina propria eosinophils were, on average, 3 times more numerous in the ascending compared with the descending colon. The result reported by Lwin et al[7] with their gastric biopsy cases was quite similar to ours in the present study.[7] Regardless, we believe that a cutoff value for eosinophil counts, which is thought to be the most important histologic criterion when rendering a diagnosis of eosinophilic gastrointestinal tract diseases, should be individualized to the different biopsy sites. The number 15 to 20 eosinophils/HPF is described often in the current textbooks[17,18] and used in most of the reports on eosinophilic gastrointestinal diseases as a cutoff value.[19–22] However, we consider that it may not be applied uniformly to all the segments of the gastrointestinal tract. Especially, in the terminal ileum and right side of the colon (C/A/T in the present study), the eosinophil count was often observed to be >20/HPF. In fact, the highest average eosinophil count was noted to be 28.60/HPF in the terminal ileum.

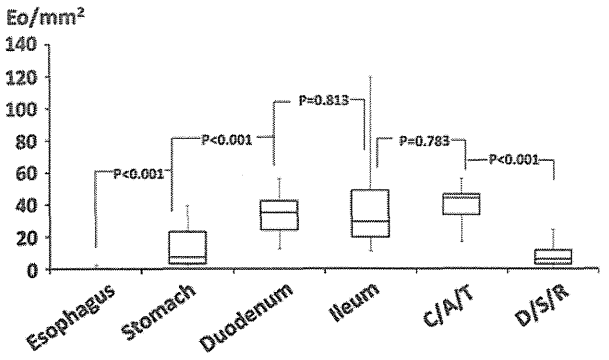

FIGURE 2. Eosinophil levels in gastrointestinal segments (Japanese, biopsy samples).

One might argue that the use of surgical resection specimens for carcinoma complicates the assessment of the normal number of eosinophils. We agree that surgical specimens are less suitable for elucidating accurate number of eosinophils than biopsy specimens, but we believe they can be used for international comparison as long as the sample collection method is strictly the same in each ethnic group. Our statistical data revealed a significant difference in eosinophil content in the esophageal intraepithelial layer at least between Japan and Hawaii, which might indicate that the racial or environmental factor has an effect on the eosinophil content as far as the esophagus is concerned. However, as described above, the study of Japanese biopsy samples detected very few eosinophils in the esophageal epithelium, with the highest average count of 2.52/mm^2 (0.60/HPF). Even the Japanese surgical samples showed a highest average count of only 7.16/mm^2 (1.70/HPF). As the eosinophil counts were so low, the statistical differences in the esophageal epithelium among the 3 groups might not be practically important when counting eosinophils. There were no significant differences in eosinophil content in the other portions of gastrointestinal tract mucosa among the 3 groups (J, JA, and W). Furthermore, the distribution patterns of eosinophils throughout the gastrointestinal tract mucosa were strikingly similar among the 3 ethnic groups. Therefore, the international comparison in the present study seems to indicate that race and environmental factors had only a little effect on the eosinophil concentration in the gastrointestinal tract mucosa, contrary to our expectation that there would be distinct statistical differences. Geographic variations in eosinophil concentration in normal colonic mucosa were studied by Pascal et al[23] in 1997. According to their study, the mean number of eosinophils was significantly higher in the southern compared with the northern United States. Their result is seemingly opposite from our study of eosinophil content in the colonic mucosa. However, as we just compared the eosinophil concentration of samples from Japan with those from Hawaii, it is possible that there is a difference between the 2 studies. Seasonal variation was not taken into account in the present study. However, interestingly, Polydorides et al[5] found that the relationship between colonic eosinophilia and seasonal allergen exposure was not significant, although mucosal eosinophils were slightly more numerous in samples obtained in April and May. Furthermore, Sorser et al[24] reported that seasons did not affect the onset of symptoms of eosinophilic esophagitis in children and adolescents. There has been no literature investigating the effect of race on eosinophil concentration in gastrointestinal tract mucosa. However, further comparisons need to be undertaken among various ethnic groups, geographic areas, and seasons.

In summary, we have elucidated the number of eosinophils in the mucosa throughout the gastrointestinal

TABLE 3. Effect of Race and Environmental Factors on Eosinophil Number in Gastrointestinal Tract (Number/mm^2)

	Mean ± SD (Range)				*P*			
	J	JA	W	JA+W	J Vs. JA	J Vs. W	JA Vs. W	J Vs. JA+W
Esophagus (IE)	0.82 ± 1.42 (0.00-7.16)	0.03 ± 0.13 (0.00-0.51)	0.36 ± 1.13 (0.00-3.7)	0.16 ± 0.70 (0.00-3.57)	0.001	0.028	0.856	< 0.001
Esophagus (SE)	5.63 ± 5.63 (0.84-23.59)	4.39 ± 4.55 (0.00-13.76)	4.48 ± 5.82 (0.00-18.34)	4.43 ± 4.96 (0.00-18.34)	0.442	0.286	0.737	0.258
Stomach	22.71 ± 16.81 (1.87-64.87)	36.35 ± 17.40 (7.13-91.21)	23.95 ± 26.68 (1.02-75.92)	31.39 ± 27.27 (1.02-91.21)	0.202	0.517	0.122	0.606
Ileum (terminal	97.05 ± 52.32 (24.01-203.88)	78.95 ± 41.14 (12.23-142.17)	70.04 ± 42.96 (0.51-178.34)	74.38 ± 41.77 (0.51-178.34)	0.379	0.141	0.310	0.606
Large intestine (C/A/T)	79.46 ± 47.03 (16.85-163.86)	73.12 ± 33.14 (19.87-109.55)	48.15 ± 43.39 (1.02-102.93)	60.6 ± 39.05 (1.02-109.55)	1.000	0.207	0.240	0.432
Large intestine (D/S/R)	38.59 ± 31.36 (2.53-106.99)	24.33 ± 20.78 (1.02-54.52)	30.67 ± 30.95 (0.00-93.76)	28.00 ± 26.66 (0.00-93.76)	0.297	0.315	0.840	0.182

IE indicates intraepithelial; SE, subepithelial (= lamina propria).

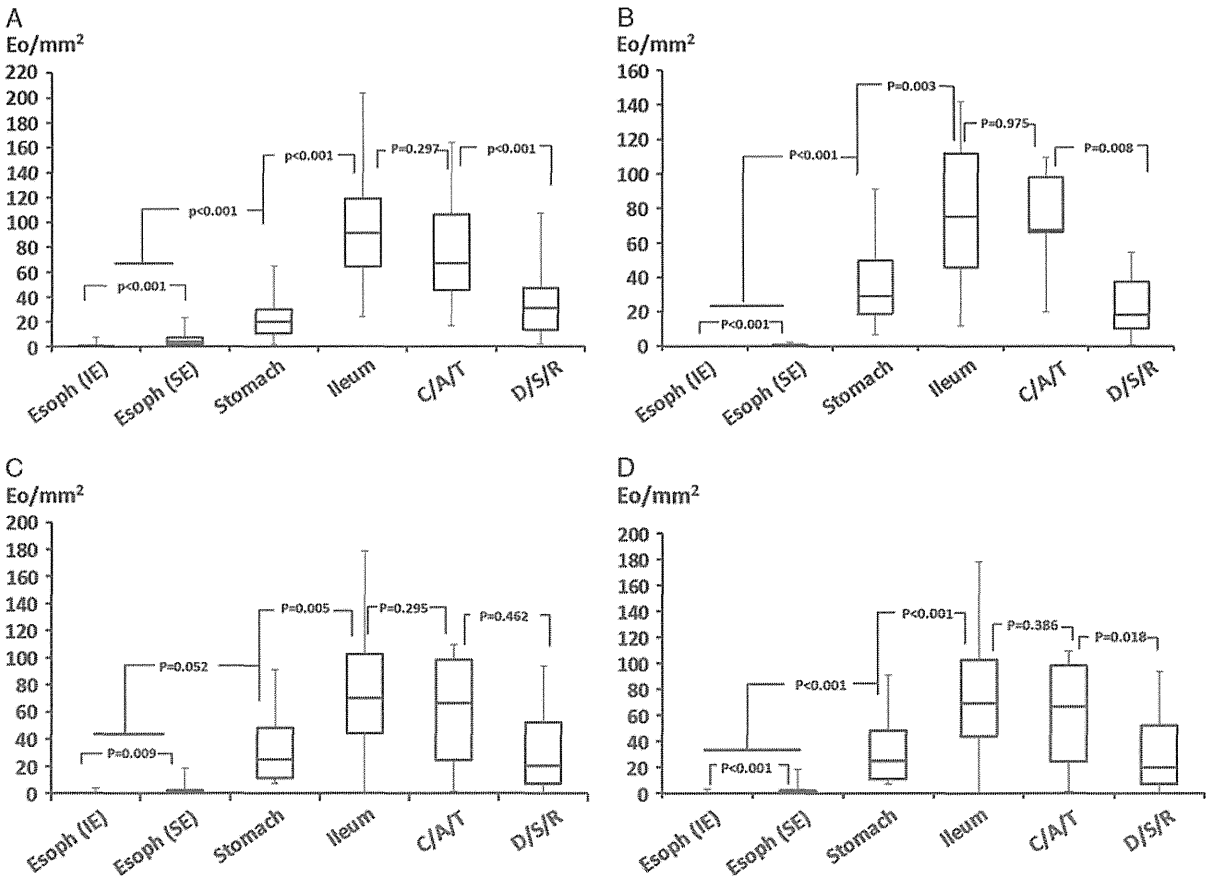

FIGURE 3. Eosinophil levels in gastrointestinal segments of the 3 ethnic groups (surgical samples). A, Japanese. B, Japanese American. C, Caucasian. D, Japanese American+Caucasian. IE indicates intraepithelial; SE, subepithelial (=lamina propria).

tract in a Japanese adult population. This is the first report of gastrointestinal tract eosinophil concentration and distribution in an Asian population. We believe that the histologic diagnosis of eosinophilic gastrointestinal tract diseases should be made on the basis of an individualized normal range of resident eosinophils. Our data suggest that race and environmental factors have only a little effect on eosinophil content at least between Japan and Hawaii, although the issue remains to be further clarified by additional investigation.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Role of milk fat globule-epidermal growth factor 8 in colonic inflammation and carcinogenesis

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Abstract
Background Milk fat globule-epidermal growth factor 8 (MFG-E8) promotes phagocytic clearance of apoptotic cells to maintain normal tissue homeostasis. However, its functions in intestinal inflammation and carcinogenesis are unknown.
Methods Experimental colitis was induced in MFG-E8 knockout (KO) and wild-type (WT) mice by dextran sodium sulfate (DSS) administration. Colon tissues were used for assessments of colitis activity and epithelial proliferation. A mouse colitis-associated cancer (CAC) model was induced by intraperitoneal injection of azoxymethane (AOM) and then the animals were given a single administration of DSS. A sporadic colon cancer model was established by repeated intraperitoneal injections of AOM. The role of MFG-E8 in epithelial proliferation with or without treatment of siRNA targeting α_v-integrin was examined in vitro using a WST-1 assay.
Results The severity of colitis in KO mice was greater than that in WT mice, while the proliferative potential of colonic epithelial cells in KO mice was lower during the regenerative phase. In both CAC and sporadic models, tumor size in KO was lower as compared to WT mice, while decreased tumor incidence was only found in the CAC model. In vitro findings showed that MFG-E8 promotes epithelial cell proliferation, and treatment with a siRNA targeting α_v-integrin reduced the proliferation of Colon-26 cells stimulated with recombinant MFG-E8.
Conclusions MFG-E8 promotes tumor growth regardless of the presence or absence of colonic inflammation, whereas colon tumor development is initiated by MFG-E8 under inflammatory conditions. These MFG-E8 functions may be dependent on integrin-mediated cellular signaling.

Keywords MFG-E8 · Inflammatory bowel disease · Intestinal inflammation · Colitic cancer · Colon cancer

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Introduction

Engulfment of apoptotic cells, an essential process to avoid release of dangerous and inflammatory mediators, is regulated by a variety of molecular mechanisms for maintaining immune homeostasis [1–5]. Milk fat globule-epidermal growth factor 8 (MFG-E8), a secreted glycoprotein, forms a

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link between phosphatidylserine (PS) on apoptotic cells and $\alpha_v\beta_3$-integrin on phagocytes for enhancing clearance of those cells [2, 3]. This glycoprotein is an essential molecule for preventing abnormal immune activation under physiological conditions [6, 7]. Deficiency or dysfunction of MFG-E8 leads to the accumulation of apoptotic cells in various organs, which is involved in the development of several immune-mediated disorders [8–12].

Apart from its function for apoptotic cell clearance, MFG-E8 also directly regulates a variety of cellular functions under various disease conditions. In particular, its anti-inflammatory effects in the intestinal tract have recently been reported [13–17]. Our previous study revealed that MFG-E8 down-regulates an inflammatory function of macrophages via $\alpha_v\beta_3$-integrin-dependent phosphorylated focal adhesion kinase (pFAK) signaling, which contributed to a reduction of intestinal inflammation in dextran sodium sulfate (DSS)- and trinitrobenzene sulfonic acid (TNBS)-induced colitis models [15–17]. In addition, other studies have demonstrated that MFG-E8 attenuates intestinal inflammation triggered by sepsis and ischemic–reperfusion (I/R) in several animal models [18–22].

In addition to an anti-inflammatory role, MFG-E8 enhances cell proliferation and migration, as well as anti-apoptosis and vascularization processes, which contribute to regeneration and repair of damaged tissues in various organs [20, 23, 24]. On the other hand, those functions are also closely associated with malignant cell growth and tumor progression. Previous studies have shown MFG-E8 overexpression in several malignancies including breast and bladder cancers, and melanoma, which stimulates tumor cell growth in an autocrine or paracrine manner [25–27]. MFG-E8 also promotes tumor cell invasion and metastasis by enhancing expression of angiogenesis factors, as well as down-regulating host tumor immunity [28–30]. However, its role in the pathogenesis of colon cancer remains largely unknown. In particular, there are no reports regarding the role of MFG-E8 in the development of colitis-associated colon cancer.

In the present study, we employed MFG-E8 knockout (KO) mice to examine the effect of MFG-E8 on colonic inflammation as well as its relationship to colon cancer development, and compared those findings to results obtained with wild-type (WT) mice. We found that a deficiency of MFG-E8 significantly reduced tumor incidence and growth in a colitis-associated cancer (CAC) model, while MFG-E8-dependent tumor growth was also confirmed in a sporadic colon cancer model. Furthermore, the proliferating potential of MFG-E8 was shown to be dependent on the cell surface expression of $\alpha_v\beta_3$-integrin on epithelial cells. These findings raise the possibility that blockade of MFG-E8 or its receptor may become a novel therapeutic option for colon cancer.

Materials and methods

Reagents

Dextran sodium sulfate (DSS, 5 kDa; Wako Pure Chemicals, Osaka, Japan), azoxymethane (AOM; Sigma, St Louis, MO, USA), recombinant mouse MFG-E8 (R&D Systems, Minneapolis, MN, USA), and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were acquired from their respective suppliers. The antibodies used were anti-mouse Proliferation Cell Nuclear Antigen (PCNA) (Dako, Tokyo, Japan), anti-human MFG-E8 antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), anti-mouse integrin alpha 5 (Abcam, Cambridge, UK) and anti-mouse Ki67 (Abcam).

Mice

$Mfge8^{-/-}$ mice with a C57BL/6 genetic background were obtained from RIKEN BRC, while WT C57BL/6N mice (males, 6–8 weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were cared for and handled in accordance with guidelines from the National Institutes of Health and Institute for Animal Experimentation of Shimane University, including housing under constant environmental conditions with circadian light–dark cycles.

Colitis induction and analysis

To produce an acute DSS colitis model, a group of mice was fed 2.5 % DSS in drinking water for 10 days, while the control group received only normal drinking water throughout the experimental period. Body weight (BW) was recorded as a parameter for colitis evaluation. After the colitis induction period, the mice were euthanized at various experimental time points and the colons were measured with a ruler on a nonabsorbent surface. Furthermore, colonic tissues were dissected for histological, real-time PCR, and enzyme immune assays.

Histological examinations

Tissues were formalin fixed and embedded in paraffin blocks. For histological examinations, 3-μm paraffin sections were stained with hematoxylin and eosin (H&E) to visualize their general morphology under a light microscope. Histological grading was evaluated as previously described [31]. In each histological examination, three different parameters were considered: severity of inflammation (based on polymorphonuclear neutrophil infiltration; 0–3: none, slight, moderate, severe), depth of injury (0–3: none, mucosal, mucosal and submucosal,

transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score for each parameter was multiplied by a factor reflecting the percentage of tissue involvement. (×1, 0–25 %; ×2, 26–50 %; ×3, 51–75 %; ×4, 76–100 %), then all values were added to a sum, with a maximum possible score of 40.

RNA extraction and real time-PCR

Total RNA was extracted from each sample using an RNeasy Protect Mini Kit (Qiagen Inc., Tokyo, Japan) and then equal amounts of RNA were reverse transcribed into cDNA using a QPCR cDNA Kit (Stratagene, La Jolla, CA, USA). All primers utilized were flanked by intron–exon junctions using the NCBI blast tool and Primer3 software (Supplementary Table 1). Quantitative real-time PCR was performed using an ABI PRISM 7700 sequence detection system with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The levels of mRNA were normalized to that of GAPDH using sequence detector software (Applied Biosystems).

Tumor induction and analysis

We utilized two models of murine colon cancer. The first model was established by AOM injection combined with DSS administration, in other words a CAC models. Mice were treated with 10 mg/kg of AOM followed by a single cycle of 2.0 % DSS over 7 days and then euthanized at 18 weeks after starting the experiment [32–34]. The second model was established by repeated AOM injections without DSS, in other words a sporadic cancer model. Mice were treated with 10 mg/kg of AOM weekly for 6 weeks and then euthanized at 31 weeks after starting the administrations [34–36]. Extirpated colons were fixed in 10 % neutral buffered formalin for 24 h and then stained with 0.2 % methylene blue (Sigma) for 15 min. Tumors were examined using a stereomicroscope at ×10 and ×40 magnifications and sizes were calculated using Scion Image for Windows (Scion Corporation). After the microscopic examination, colon tissues were embedded in paraffin blocks and stained with hematoxylin and eosin (H&E) for histology.

Immunohistochemistry

Mouse colonic tissue samples were used for detection of PCNA and Ki67. To evaluate MFG-E8 expression in human colonic mucosa, multiple endoscopic biopsy specimens were obtained from both active and inactive mucosa of UC patients. In addition, human colon cancer and adenoma tissue samples were obtained by surgery or endoscopic resection. Immunohistochemistry was performed using formalin-fixed paraffin-embedded blocks. After deparaffinization, endogenous peroxidase activity was blocked with Peroxidase-Blocking Solution (Dako) for 10 min. Sections (5 μm thick) were washed with PBS and incubated with an anti-human MFG-E8 antibody (Santa Cruz Biotechnology) at room temperature for 30 min. After washing again with PBS, the bound antigen–antibody complex was detected using the ChemMate™ DAKO EnVision™ Detection Kit (DAKO). The study protocols were approved by the ethics committee of Shimane University Faculty of Medicine.

Cell culture

The mouse colon cancer cell line Colon-26 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). We previously reported that Colon-26 cells express $\alpha_v\beta_3$-integrin and respond well to treatment with rMFG-E8 in vitro [15], thus they were used for the present in vitro assays. Cells were seeded at 2×10^5 cells/ml, and grown in RPMI 1640 medium (Invitrogen) and penicillin–streptomycin–amphotericin B (Invitrogen), and then maintained at 37 °C in 5 % CO_2 in a humidified incubator.

Cell counting assay

Cells were seeded into 24-well plates and harvested 24 h later using trypsinization. Then 100-μl cell suspensions were subjected to counting with a Neubauer hemocytometer (Erma, Tokyo, Japan).

WST-1 assay

A 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) cell proliferation assay is based on enzymatic cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases present in viable cells. WST-1 assays of Colon-26 cells were performed as described in the product manual (Roche, Mannheim, Germany). Formazan dye produced by viable cells was quantified by measuring absorbance at a wavelength of 450 nm.

RNA interference and transfection

Colon-26 cells were cultured in six-well plates (2×10^5 cell/ml) and then custom siRNAs (Qiagen, Valencia, CA, USA) targeting the mouse α_v integrin gene were transfected (10 nM/well) according to the manufacturer's protocol. After transfection, cells were harvested using trypsinization. The efficiency of target gene knockdown was assessed by real-time PCR and the results were

compared to those obtained with the negative control siRNA-transfected condition.

Statistical analysis

All results are expressed as the mean with the standard error of the mean (SEM) or as a range when appropriate. Student's t test was used as appropriate to examine significant differences. p values <0.05 were considered to be significant. All statistical analyses were performed using statistical analysis software (SPSS, version 12.0 for the PC; SPSS Japan, Inc.).

Results

BW change and colon morphology of MFG-E KO mice under physiological conditions

A previous study showed that MFG-E8 KO mice spontaneously develop lupus-like autoimmune nephritis due to impaired phagocytosis of apoptosis cells [9]. However, little is known regarding the morphology of the intestinal tract in KO mice under physiological conditions. In this regard, we first examined age-related changes in BW (5–30 weeks), and colon length and histology (6 and 30 weeks) in both KO and WT mice without inflammatory induction, and did not detect any differences for these parameters between those groups (Fig. 1a–e). In addition, the potential of epithelial proliferation was investigated in histological sections by PCNA staining. The number of PCNA-positive epithelial cells in KO mice was higher than that in WT mice (Fig. 1f, g).

MFG-E8 protects against DSS-induced colonic inflammation

We recently reported that DSS-induced colonic inflammation was severe in MFG-E8 KO mice [16]. In the present study, we modified the experimental design and re-examined the influence of MFG-E8 deficiency on DSS-induced colitis, with those results shown in Fig. 2. The deficiency of MFG-E8 exacerbated several colitis parameters including BW loss, histological score, and colonic expression of inflammatory cytokines, which confirmed results recently reported by our group.

Low incidence and growth of CAC in MFG-E8 KO mice

It has been reported that chronic colitis leads to development of CAC [37–39]. Notably, CAC more easily develops as colonic inflammation becomes severe. Since MFG-E8 KO mice show severe colitis (Fig. 2), we speculated that they might be more susceptible to the development of CAC than WT mice. A mouse CAC model was induced by intraperitoneal injection of AOM and then the mice were given a single cycle administration of DSS (Fig. 3a). Representative images of colon tumors and their histological appearance are shown in Fig. 2b. Contrary to our speculation, the average number of colon tumors in the KO mice was significantly lower than that in the WT mice, while the average tumor size per mouse was also lower in KO mice (Fig. 3c). These findings suggest that a lack of MFG-E8 reduces inflammation-associated tumor development as well as tumor growth even in the presence of severe colitis.

Increased expression of MFG-E8 and its association with epithelial cell proliferation

To confirm the role of MFG-E8 in CAC development, we established DSS-induced colitis in WT and KO mice (Fig. 4a) and investigated the time course changes of MFG-E8 expression in colonic tissues in the mouse models. Colonic expression of MFG-E8 was significantly increased in the WT mice during the regeneration phase of DSS-induced colitis (Fig. 4b). Next, we performed PCNA and Ki67 staining of colonic histological sections to access proliferation of epithelial cells in WT and KO mice, with representative images of stained colonic PCNA and Ki67 tissues shown in Fig. 4c and Supplementary Figure 1a (3 weeks). The prevalence of PCNA- and Ki67-positive epithelial cells was significantly greater in the WT mice as compared to the KO mice (Fig. 4d; Supplementary Figure 1b).

Decreased growth of colon tumors in sporadic colon cancer model of MFG-E8 KO mice

Next, we examined whether a lack of MFG-E8 has an influence on tumor incidence and growth in a sporadic colon cancer model. This model was established by repeated AOM injections without DSS administration (Fig. 5a) and then the mice were euthanized at 31 weeks. Representative images of colon tumors and their histological appearance are shown in Fig. 5b and c. Although we did not find significant differences regarding the number of tumors between the KO and WT mice, the average tumor size per mouse was significantly lower in the former (Fig. 5d).

Increased expression of MFG-E8 in inflammatory colonic mucosa of UC patients

We performed immunohistochemical analysis using colonic tissue sections of UC patients to confirm MFG-E8

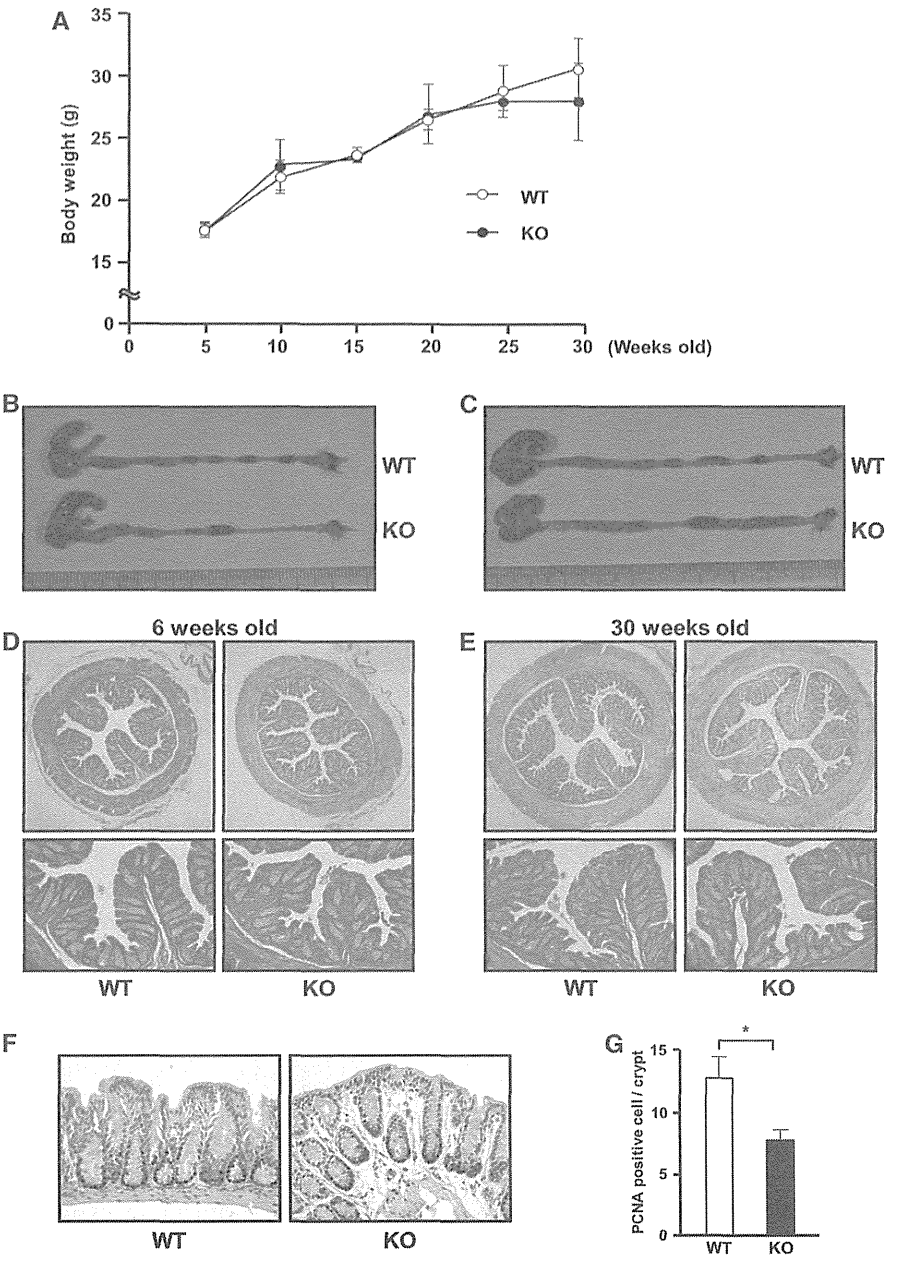

Fig. 1 The natural course of MFG-E8 KO mice under physiological conditions was similar to that of WT mice. **a** Age-related BW changes (5–30 weeks old) in KO ($n = 4$) and WT ($n = 4$) mice. Representative images show colon length (**b** 6 weeks, **c** 30 weeks) and histology (**d** 6 weeks, **e** 30 weeks), and immunohistochemistry for PCNA (30 weeks). **g** Average numbers of PCNA-positive epithelial cells (KO, $n = 6$; WT, $n = 6$)

localization, which revealed its presence in lamina propria mononuclear cells (Fig. 6A). MFG-E8 expression was significantly higher in active mucosa as compared to inactive mucosa (Fig. 6B).

MFG-E8 expression in human colon cancer cells

Previous studies have revealed that several sporadic cancers express abundant MFG-E8, which contributes to the growth or invasion of tumors. However, nothing is known regarding MFG-E8 expression in sporadic colon cancer. In the present study, we investigated MFG-E8 expression in surgically resected human advanced colon cancer tissues. Representative images of immunohistochemistry findings are shown in Fig. 6. MFG-E8 expression was clearly observed in colon cancers cells (Fig. 6C-a, C-b), whereas that was faintly seen in non-tumorous lesions (Fig. 6C-c). Immunoreactive signals of MFG-E8 were also observed in mononuclear cells that had infiltrated around the cancer cells (Fig. 6C-d). We also evaluated MFG-E8 expression in 17 advanced colon cancer specimens and found that the percentage of cases with positive findings

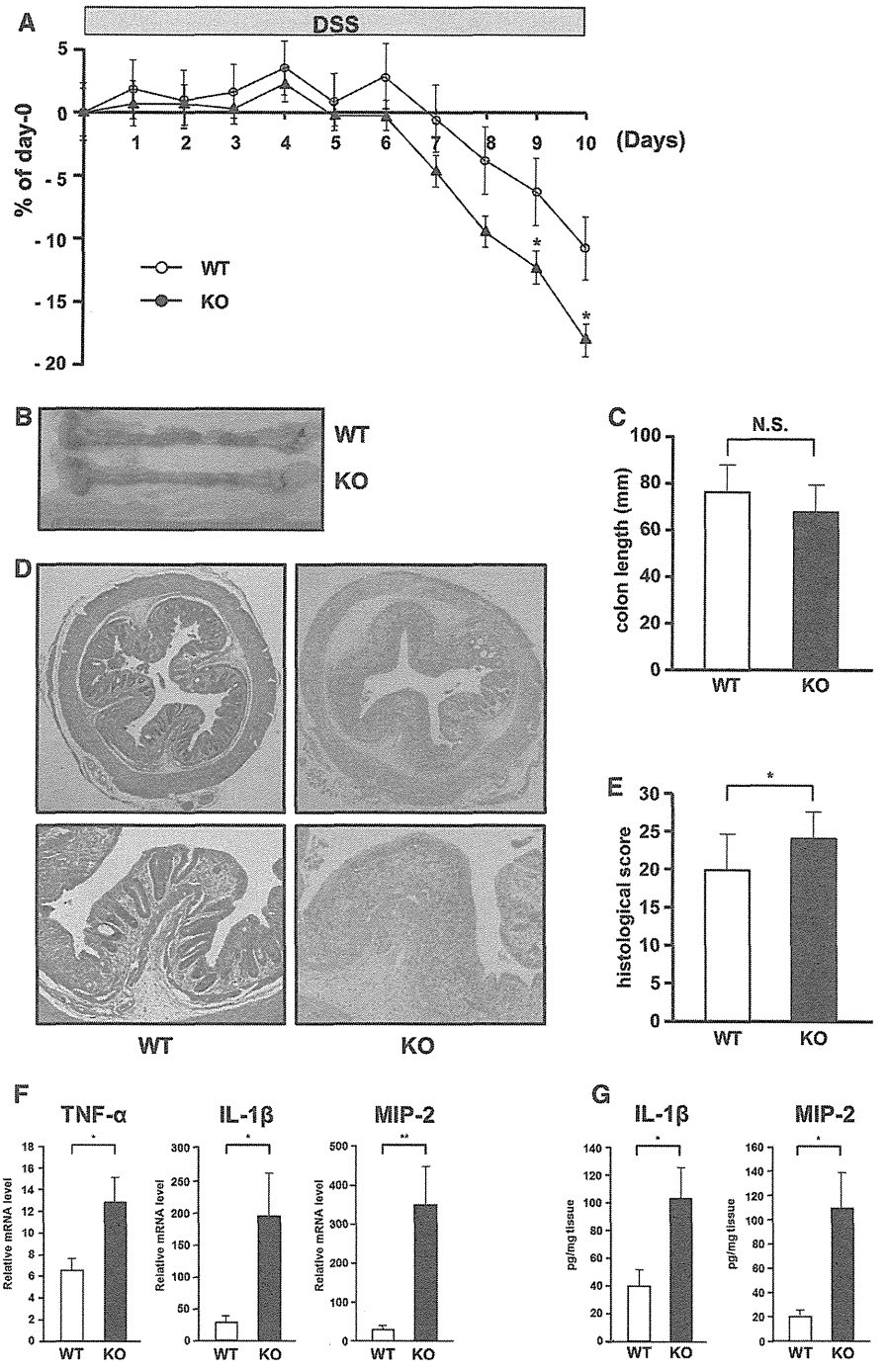

Fig. 2 DSS-induced colitis was found to be exacerbated in MFG-E8 KO mice. Experimental colitis models were established by administering a 2.5 % DSS solution in drinking water for 10 days (WT, $n = 7$; KO, $n = 7$). **a** BW changes in WT and KO mice with DSS-induced colitis. **b** Representative image showing colon lengths. **c** Average colon lengths. **d** Representative images showing inflamed colon histology. **e** Average histological scores. **f**, **g** Cytokine mRNA and protein levels in colonic tissues. $*p < 0.05$ vs. WT, $**p < 0.01$ vs. WT

was 73.0 %. Notably, abundant expression was observed in the deeper invasive parts of the cancer tissues. In addition, we performed immunostaining for detection of MFG-E8 in adenoma (26 cases) and early cancer (23 cases) tumor tissues, and found that its expression rate in those was 18.2 and 57.0 %, respectively. Thus, MFG-E8 expression in samples from human colon adenomas was shown to gradually increase from early to advanced cancer.

MFG-E8 stimulates epithelial cell proliferation via $\alpha_v\beta_3$-integrin

Based on the results of our chimeric mice experiments, we next investigated whether MFG-E8 directly induces proliferation of colonic epithelial cells (Colon-26 cells) in vitro. Treatment with recombinant mouse MFG-E8 (rMFG-E8) significantly stimulated proliferation of Colon-

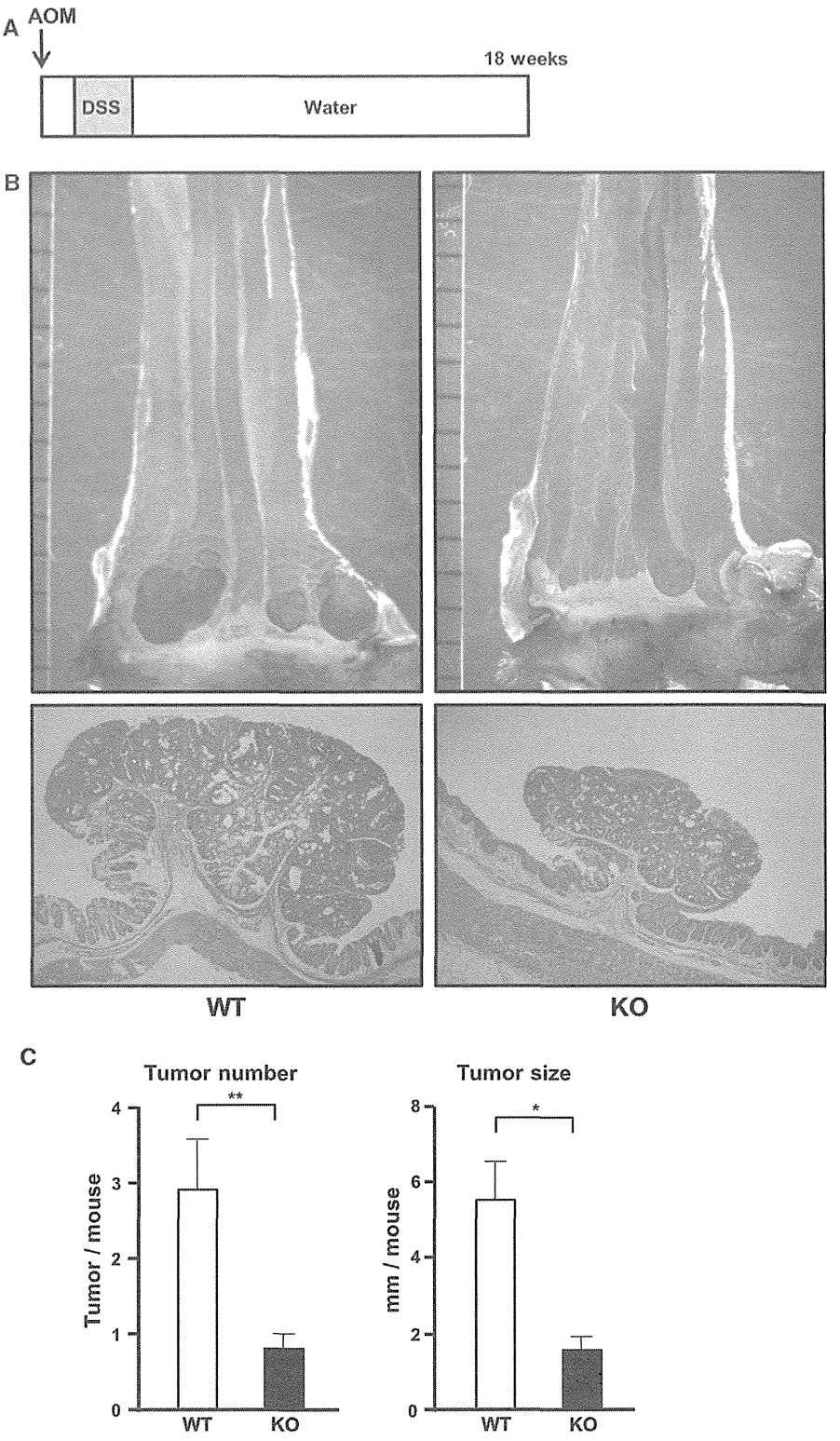

Fig. 3 In the CAC model, MFG-E8 KO mice developed fewer tumors and showed reduced tumor size as compared to the WT mice. **a** Schematic overview of CAC model. Mice were injected with AOM (10 mg/kg) in an intraperitoneal manner, followed by a single cycle of DSS (2.0 %, 7 days) in drinking water and euthanized at 18 weeks. **b** Tumor morphology shown by stereoscopic microscopy (*upper* 0.2 % methylene blue staining) and histology (*lower* hematoxylin and eosin staining). **c** Average number and size of tumors per mouse in WT ($n = 12$) and MFG-E8 KO ($n = 10$) mice. $^{**}p < 0.01$ vs. WT, $^{*}p < 0.05$ vs. WT

26 cells (Fig. 7a), which was confirmed by the results of WST-1 assay (Fig. 7b). Finally, since the biological functions of MFG-E8 are dependent on the cell surface receptor $\alpha_v\beta_3$-integrin, we also examined its role in MFG-E8-induced epithelial proliferation. Treatment with a neutralizing antibody (Fig. 7c) or siRNA (Fig. 7d) targeting α_v-