titative real-time PCR) were defined as positive for CMV infection.

Statistical Analysis

Categorical and continuous data were compared using a 2-tailed Fisher exact test and Mann–Whitney *U*-test. CMV-positive patients were compared with CMV-negative patients for different parameters (age, DAI score, extent of disease, endoscopic score of DAI, Matts grade, endoscopic index of Rachmilewitz, ratio of patients undergoing colectomy, and treatment). A P-value <0.05 was considered statistically significant.

RESULTS

The clinical characteristics of the 30 patients are summarized in Table 2. The mean age of the 30 patients was 40.8 ± 17.6 years (range 16–73 years), and the mean DAI score was 9.5 ± 1.4. The extent of the disease was proctitis (3.3%), left-sided colitis (23.3%), and pancolitis (73.3%). The mean endoscopic DAI score was 2.3 ± 0.7, the mean Matts grade was 3.0 ± 0.8, and the mean endoscopic index score was 9.2 ± 2.4 (Table 2).

Of the 30 patients, 23 (76.7%) had been treated with corticosteroids, 6 (20.0%) with azathioprine, 7 (23.3%) with tacrolimus, and 2 (6.7%) with leukocytapheresis when visiting our institution. Six patients (20.0%) received colectomy during the observation period (Table 2, Fig. 1).

CMV-DNA was detected in the colonic tissues of 17 patients (56.7%) (4 with left-sided colitis, 13 with pancolitis) (Table 3). Notably, in all positive cases CMV-DNA was detected only in the inflamed colonic mucosa and not in the noninflamed mucosa. As a control, we examined CMV-DNA in the inflamed mucosa of 4 patients with UC who were in clinical remission with immunosuppressive therapies. CMV-DNA was not detected in the inflamed mucosa of any of these patients (data not shown). On the other hand, CMV antigenemia and histologic examination were positive in only 3 (17.6%) and 1 (5.9%) of the 17 patients positive for CMV-DNA in the colonic mucosa, respectively, and none of the patients negative for CMV-DNA in the colonic mucosa was positive for either CMV antigenemia or histologic examination.

A comparison of differences in age, DAI score, disease extent, ratio of patients undergoing colectomy, and the endoscopic score between CMV-DNA-positive and -negative patients revealed no significant differences between the 2 groups, although the number of patients who received colectomy tended to be greater in CMV-DNA-positive patients than in -negative patients (Table 4).

Moreover, a comparison of difference in treatment between CMV-DNA-positive and -negative patients also revealed no significance difference between the 2 groups. However, the number of patients treated with corticosteroid tended to be greater than in CMV-DNA-positive patients than in -negative patients (Table 5).

Of the 17 CMV-DNA-positive patients, 12 (70.6%) were treated with ganciclovir daily for 2 weeks (Fig. 1). CMV-DNA in the colonic mucosa became negative in all patients that received antiviral therapy. Four patients (33.3%) went into remission following antiviral therapy only. Although 7 patients (58.3%) were improved after antiviral therapy, the underlying UC remained active. These patients were treated with additional granulocytapheresis (GCAP) using an Adacolumn (Japan Immunoresearch Laboratories, Takasaki, Japan) or additional tacrolimus after their CMV-DNA became negative. Six (50.0%) of them went into remission, but 1 (8.3%) patient did not and therefore received a colectomy.

TABLE 2. Clinical Characteristics of 30 Patients with UC Refractory to Immunosuppressive Therapies

Age (mean ± SD)		40.8±17.6
Sex (M/F)		14/16
DAI score		9.5 ± 1.4
Extent of disease	Proctitis	1 (3.3)[a]
	Left-sided	7 (23.3)[a]
	Pancolitis	22 (73.3)[a]
Endoscopic score of DAI		2.3 ± 0.7
Matts grade		3.0 ± 0.8
Endoscopic index of Rachmilewitz		9.2 ± 2.4
Treatment on admission	Corticosteroid (CS)	23 (76.7)[a]
	Corticosteroid alone	14 (60.9)[b]
	With azathioprine	5 (21.7)[b]
	With tacrolimus	1 (4.3)[b]
	With LCAP	2 (9.1)[b]
	With GCAP	1 (4.3)[b]
	Azathioprine (AZA)	6 (20.0)[a]
	Azathioprine alone	1 (16.7)[c]
	With corticosteroid	5 (83.3)[c]
	Tacrolimus	7 (23.3)[a]
	Tacrolimus alone	5 (71.4)[d]
	With corticosteroid	1 (14.3)[d]
	With infliximab	1 (14.3)[d]
	LCAP	2 (6.7)[a]
	With corticosteroid	2 (100.0)[e]
Ratio undergoing colectomy		6 (20.0)[a]

Number of patients is shown. Age, DAI Score, Endoscopic Score of DAI, Matts Grade, and Endoscopic Index of Rachmilewitz are presented as mean SD. LCAP, leukocytapheresis

[a] Values in parentheses are percentages of all 30 patients.
[b] Values in parentheses are percentages of patients treated with CS.
[c] Values in parentheses are percentages of patients treated with AZA.
[d] Values in parentheses are percentages of patients treated with tacrolimus.
[e] Values in parentheses are percentages of patients treated with LCAP.

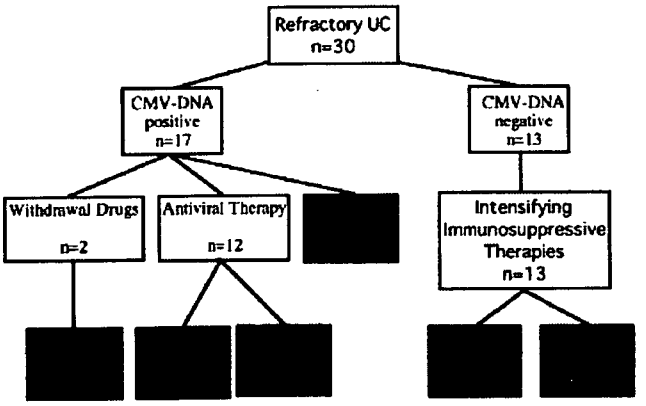

FIGURE 1. Clinical course of 30 patients with UC refractory to immunosuppressive therapy. CMV-DNA in inflamed mucosa was positive in 56.7% (17/30) of patients with UC, and negative in 43.3% (13/30) of patients with UC. Shaded rectangular columns with double lines show final outcomes.

Eventually, 10 (83.3%) of the 12 patients that received antiviral therapy went into remission and the remaining 2 patients (16.7%) received a colectomy. Three (17.6%) of the remaining 5 patients positive for CMV-DNA required urgent colectomy without receiving antiviral therapy, and 2 (11.8%) achieved remission by withdrawal of the immunosuppressive drugs.

Of the 13 CMV-DNA-negative UC patients, 12 (92.3%) went into remission after treatment with more intense immunosuppressive therapies. Only 1 patient (7.7%) was refractory to additional immunosuppressive therapies and finally required a colectomy.

DISCUSSION

In the present study, we applied quantitative real-time PCR for the diagnosis of active CMV infection in the colonic mucosa of 30 patients with UC refractory to immunosuppressive therapies. Using this method the detection rate of CMV

TABLE 3. Detection Rate of CMV-DNA in the Colonic Mucosa of UC Patients Refractory to Immunosuppressive Therapies

	CMV-DNA in Inflamed Mucosa	CMV-DNA in Noninflamed Mucosa	Antigenemia	IHC
CMV infection				
Postitive	17 (56.7%)	0	3 (17.6%)	1 (5.9%)
Negative	0	0	0	0
Total	17	0	3	1

Number of patients is shown. Values in parentheses are percentages of the total number of patients (n = 30).

TABLE 4. Comparison of Clinical Parameters between UC Patients with and without Detectable CMV-DNA in the Inflamed Mucosa

	CMV-DNA-positive N = 17	CMV-DNA-negative N = 13	P-value
Age	44.1 ± 16.3	36.5 ± 18.9	0.247
DAI score	9.8 ± 1.2	9.2 ± 1.6	0.206
Extent of disease			
Proctitis	0 (0.0)	1 (7.7)	0.245
Left-sided	4 (23.5)	3 (23.1)	0.977
Pancolitis	13 (76.5)	9 (69.2)	0.657
Endoscopic score of DAI	2.4 ± 0.7	2.1 ± 0.6	0.194
Matts grade	3.1 ± 0.8	2.9 ± 0.8	0.687
Endoscopic index of Rachmilewitz	9.5 ± 2.4	8.8 ± 2.4	0.444
Ratio undergoing colectomy	5 (29.4)	1 (7.7)	0.196

Number of patients is shown. Age, DAI Score, Endoscopic Score of DAI, Matts Grade, and Endoscopic Index of Rachmilewitz are presented as mean ± SD. Values in parentheses are percentages of the total number of patients either positive or negative for CMV-DNA in the inflamed mucosa.

TABLE 5. Comparison of Treatment between UC Patients with and without Detectable CMV-DNA in the Inflamed Mucosa

Treatment	CMV-positive n = 17	CMV-negative n = 13
Corticosteroid (CS)	15 (65.2)[a]	8 (34.8)[a]
Corticosteroid only	10	4
With azathioprine	3	2
With tacrolimus	1	0
With LCAP	1	1
With GCAP	0	1
Azathioprine	3 (50.0)[b]	3 (50.0)[b]
Azathioprine alone	0	1
With corticosteroid	3	2
Tacrolimus	3 (42.9)[c]	4 (57.1)[c]
Tacrolimus alone	1	4
With corticosteroid	1	0
With infliximab	1	0
LCAP	1 (50.0)[d]	1 (50.0)[d]
With corticosteroid	1	1

Number of patients is shown. There is no significant difference in treatment between CMV-DNA-positive and -negative patients.
[a] Values in parentheses are percentages of patients treated with CS.
[b] Values in parentheses are percentages of patients treated with AZA.
[c] Values in parentheses are percentages of patients treated with tacrolimus.
[d] Values in parentheses are percentages of patients treated with LCAP.

infection tended to be higher than when using other conventional methods such as CMV antigenemia and histologic examination. Moreover, a high remission rate was achieved in UC patients refractory to immunosuppressive therapies by applying either antiviral therapy or modulating immunosuppressive therapies according to the results of the quantitative real-time PCR for CMV-DNA in the inflamed colonic mucosa. Thus, our real-time PCR method for detecting CMV-DNA appears to be more useful than conventional modalities for diagnosing active CMV infection in patients with UC refractory to immunosuppressive therapies.

Quantitative real-time PCR revealed that CMV-DNA was positive in the inflamed colonic mucosa of 56.7% (17/30) of our UC patients, whereas CMV antigenemia and histologic examination were positive in only 17.6% (3/17) and 5.9% (1/17) of the patients positive for CMV-DNA, respectively. Thus, the detection rate of CMV infection by quantitative real-time PCR far exceeded that by CMV antigenemia and histologic examination. Several methods are used to diagnose CMV infection, including histologic examination, CMV antigenemia, and PCR assay.[7–9] Among them, CMV antigenemia and PCR assay using whole blood potentially reflect the reactivation of CMV in the whole body, but does not necessarily indicate CMV infection in the colonic mucosa. Indeed, there are several reports of reactivation of CMV in the plasma of patients with collagen disease and AIDS without gastrointestinal involvement of CMV infection.[7,19] Reactivation of CMV in the plasma does not reflect the involvement of CMV infection in UC.[20] Histologic examination is often considered the "gold standard" for diagnosing CMV infection in the gastrointestinal tract.[21] Its sensitivity for diagnosis, however, ranges from 10%–87%, and moreover, 37.5% of patients with gastrointestinal CMV disease fail to demonstrate any inclusions.[21] To overcome such low sensitivity, IHC with monoclonal antibodies was developed. The sensitivity for detecting CMV infection with IHC ranges from 78%–93%.[21] The sensitivity and specificity of CMV antigenemia for detecting CMV infection are 60%–100% and 83%–100%, respectively.[21] The present data, however, indicate that the detection rates of CMV infection by those established methods are lower than previously reported. In contrast, we found a significantly higher detection rate of CMV-DNA in the inflamed colonic mucosa by our quantitative real-time PCR system than by conventional modalities such as histologic examination and CMV antigenemia. These data strongly suggest that our quantitative real-time PCR for detecting CMV-DNA in the inflamed mucosa is very useful for diagnosing active CMV infection in patients with UC refractory to immunosuppressive therapies. It should be emphasized that none of the patients that were positive for CMV-DNA in the inflamed colonic mucosa were positive for CMV-DNA in the noninflamed mucosa. Thus, the high sensitivity of our method for detecting CMV infection is likely to be due to the sampling of the inflamed mucosa for the assay.

CMV is present in its latent form in most healthy subjects.[18] Therefore, we might expect low specificity for diagnosing active CMV infection when using a sensitive PCR method, because it is possible that sensitive PCR will detect CMV-DNA in subjects with a latent CMV infection. In this respect, we observed that CMV-DNA was detected only in the inflamed colonic mucosa and not in the noninflamed colonic mucosa. Moreover, by using biopsy specimens from the inflamed colonic mucosa, 12 of our 17 CMV-DNA-positive patients achieved remission by either antiviral therapy or withdrawing immunosuppressive therapies, whereas 12 of 13 CMV-DNA-negative patients achieved remission by intensifying the immunosuppressive therapies. In addition, none of the patients negative for CMV-DNA were positive based on either histologic examination or CMV antigenemia. Taken together, these findings suggest that both the sensitivity and specificity of our quantitative real-time PCR for diagnosing active CMV infection are high, and indeed, the findings were useful for making an appropriate decision regarding whether the immunosuppressive therapies should be intensified or tapered.

Endoscopy is a useful modality for diagnosing CMV infection when the characteristic findings such as deep ulceration are observed.[22] The endoscopic findings in CMV-positive colitis, however, vary,[21,23] and thus it might be difficult to distinguish CMV infection from severe UC. Sakamoto et al[24] reported that no specific endoscopic findings were observed in UC patients with concomitant CMV infection. We also evaluated whether endoscopic findings was useful for early detection of CMV infection using endoscopic score in the present study. Our data revealed no significant difference in endoscopic score according to 3 different indexes between the CMV-DNA-positive and -negative patients. Based on both the previous reports and the present report, the significance of endoscopic findings for diagnosing CMV in patients with UC remains unclear. Hommes et al[25] proposed a mechanism of CMV replication and activation in the intestinal tissue during active inflammatory bowel disease and classified the findings into 3 stages (initiation, reactivation, and consolidation). According to their proposal, the stage at which we detected CMV-DNA in the inflamed colonic mucosa of patients with UC might correspond to the initiation or reactivation stage prior to the occurrence of characteristic endoscopic findings in CMV colitis. Thus, one reason for the lack of a significant difference in the endoscopic score between the CMV-DNA-positive and -negative patients in our study might be due to the detection of CMV infection at an early stage.

An interesting observation in our study is that, as noted above, CMV-DNA was detected only in the inflamed colonic mucosa, and not in the noninflamed mucosa by quantitative

real-time PCR. Hahn et al[26] reported that proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α induce the reactivation of CMV. Hommes et al[25] also reported that those proinflammatory cytokines induce the migration of monocytes to the inflammatory sites of the colonic mucosa and promote their differentiation into macrophages, which have a role in supporting active replication of CMV as CMV reservoir cells. Thus, it might be that, in patients with UC, CMV is more easily reactivated in the inflamed mucosa than in the noninflamed mucosa.

The therapeutic strategy for UC patients with concomitant CMV infection is a very important issue. In this study, 10 (83.3%) of the 12 CMV-DNA-positive patients went into remission after applying antiviral therapy and modulating immunosuppressive therapies. Of the 13 CMV-DNA-negative UC patients, moreover, 12 (92.3%) went into remission after treatment with more intense immunosuppressive therapies. At present, in UC patients refractory to immunosuppressive therapies we first perform quantitative real-time PCR using inflamed mucosa. In CMV-DNA-positive cases, antiviral therapy should be applied promptly and immunosuppressive therapies should be tapered. After CMV-DNA became negative, immunosuppressive therapies could be intensified. On the other hand, in CMV-DNA-negative cases immunosuppressive therapies could be intensified. Thus, clinical outcome in this study revealed that our quantitative real-time PCR using inflamed mucosa was useful for making a decision of treatment for patients with UC refractory to immunosuppressive therapies.

In conclusion, our use of quantitative real-time PCR for detecting CMV-DNA in inflamed mucosa was very useful for the early and accurate diagnosis of active CMV infection in patients with UC refractory to immunosuppressive therapies, enabling prompt and appropriate treatment. Further studies are required to determine whether this method will contribute to improving the prognosis of UC complicated by active CMV infection.

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GASTROENTEROLOGY

Open label trial of clarithromycin therapy in Japanese patients with Crohn's disease

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antibiotics, clarithromycin, Crohn's disease, immunomodulator, luminal bacteria.

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Abstract

Background and Aim: The pathogenesis of Crohn's disease is unclear, but many studies suggest that luminal bacteria play an important role in chronic intestinal inflammation in patients with this condition. Clarithromycin is a macrolide antibiotic with immunomodulatory activity. The aim of this study was to evaluate the effect of clarithromycin therapy in Japanese patients with Crohn's disease.

Methods: Fourteen patients with active Crohn's disease (12 with ileocolonic, one with colonic, one with small bowel type) were treated with oral clarithromycin 200 mg twice daily for 4 weeks. Patients who showed a clinical response within 4 weeks continued the therapy for up to 24 weeks. Four patients also received azathioprine. Clinical activity was assessed with the Crohn's Disease Activity Index (CDAI) at entry and at 4, 12, and 24 weeks after starting clarithromycin.

Results: The mean CDAI score at entry was 343.5. Within 4 weeks, eight (57.1%) of the 14 patients showed clinical improvement, and five (35.7%) of the eight patients achieved remission. All of those eight patients continued clarithromycin therapy after 4 weeks, and six (42.9%) were in clinical remission at 12 weeks. Of the 14 total patients, four (28.6%) continued clarithromycin for more than 24 weeks, and have remained in remission. Patients who received azathioprine concomitantly had a better response to clarithromycin therapy. No severe side-effects were observed during the study period.

Conclusions: This open label study showed encouraging results of clarithromycin therapy in Japanese patients with active Crohn's disease.

Introduction

The etiology of Crohn's disease remains unclear, and both genetic and environmental factors seem to be involved in its pathogenesis. Recent data suggest that indigenous bacterial flora play an important role in the initiation and perpetuation of chronic intestinal inflammation in patients with Crohn's disease.[1] Therefore, antibiotics are considered as one of the basic therapies.[2] Generally, metronidazole and ciprofloxacin are the antibiotics most often used to treat mild to moderate luminal or fistulizing Crohn's disease.[3] In a double blind, placebo-controlled trial of metronidazole in Crohn's disease, there was a significant reduction in Crohn's Disease Activity Index (CDAI) scores and C-reactive protein levels in the metronidazole-treated group compared with placebo.[4] A controlled 6-month trial of ciprofloxacin in Crohn's disease demonstrated a significant reduction in CDAI scores in the ciprofloxacin-treated group.[5] In combination therapy with metronidazole and ciprofloxacin for 10 weeks, 55 of 72 patients (76%) showed a clinical response and 49 of 72 patients (68%) achieved clinical remission.[6] This combination therapy was also effective for Japanese patients with Crohn's disease.[7]

Clarithromycin is a broad-spectrum macrolide antibiotic. Macrolides, such as clarithromycin and azithromycin, bind to the 50S subunit of the 70S bacterial ribosome, and thereby inhibit bacterial protein synthesis. They accumulate in extremely high levels within macrophages and have prolonged intracellular half-lives.[8] These traits enhance their efficacy against intracellular organisms. Similar to other macrolides, clarithromycin acts not only as an antibiotic but also as an immunomodulator, which can enhance macrophage proliferation, phagocytosis, chemotaxis, and cytocidal activity.[9] Clarithromycin suppresses nuclear factor (NF)-κB activation in response to tumor necrosis factor (TNF)-α in monocytes and lymphocytes,[10] and thus represses production of cytokines such as interleukin (IL)-8, TNF-α, and IL-1.[11–13]

There are several reports of open label studies of treatment for Crohn's disease with clarithromycin,[14–20] although its therapeutic results are still inconclusive. The aim of our trial is to evaluate the

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effectiveness of clarithromycin in Japanese patients with active Crohn's disease.

Methods

Patients

Fourteen patients (10 male, 4 female) with active Crohn's disease (CDAI ≥ 150 for more than 3 weeks) were enrolled in this study. The diagnosis of Crohn's disease was confirmed by clinical, radiological, endoscopic, and histological findings. The study was approved by the ethics committee of Kyoto University Hospital. Informed consent was obtained from all patients. The baseline characteristics of the patients are shown in Tables 1 and 2. All the patients were older than 20 years of age (mean 33.6, range 26–48 years), with ileocolonic ($n = 12$), colonic ($n = 1$), or small bowel-type ($n = 1$) Crohn's disease. At entry, the mean CDAI score was 343.5 (range 164.9–529.2).

Four patients (28.6%) were receiving azathioprine. The dose of azathioprine had been unchanged for more than 3 months before starting clarithromycin. Only one patient (7.1%) was receiving corticosteroids (7.5 mg/day) for 6 weeks prior to clarithromycin therapy. Eleven patients (78.6%) were receiving 5-aminosalicylate or sulfasalazine, and 11 patients (78.6%) were receiving elemental diet therapy. These drugs and therapy were kept unchanged during this study except in the one patient receiving steroid therapy in whom the dose was reduced as he clinically improved.

Table 1 Baseline characteristics of 14 patients with Crohn's disease

Characteristic	*n* (%)
Sex	
Male	10 (71.4)
Female	4 (28.6)
Disease location	
Colonic	1 (7.1)
Ileocolonic	12 (85.7)
Small bowel only	1 (7.1)
Previous resection	4 (28.6)
Treatment	
Elemental diet	11 (78.6)
5-Aminosalicylate, SASP	11 (78.6)
Steroids	1 (7.1)
Azathioprine	4 (28.6)
Intestinal complication	
Stenosis	5 (35 7)
Abscess	2 (14.3)
Fistula	3 (21.4)

SASP, sulfasalazine.

Table 2 Mean characteristics at entry of 14 patients with Crohn's disease

Characteristic	Mean	Median	Range
Age (years)	33.6	32	26–48
Duration of disease (years)	12 5	11 5	1–25
Crohn's Disease Activity Index	343 5	361 9	164 9–529.2
C-reactive protein (mg/L)	27.2	16	1–94

Study design

Patients received clarithromycin 200 mg twice daily for at least 4 weeks while they continued baseline treatment. There have been several reports that long-term administration with 400 mg/day of clarithromycin was well tolerated, safe, and effective for Japanese patients with chronic bronchitis or sinusitis.[21] Therefore, we used the same dose in our clinical trial.

Four weeks after starting clarithromycin therapy, we evaluated the CDAI, and decided whether or not clarithromycin therapy should be continued. The responders continued clarithromycin therapy for 24 weeks. If patients showed no clinical response or relapsed, clarithromycin therapy was stopped and alternative treatment was started. In this study, four patients continued on clarithromycin therapy for a median of 55 weeks (range 36–76) to study the long-term outcomes and side-effects.

Clinical activity was assessed with the CDAI and C-reactive protein levels at entry, and at 4, 12, and 24 weeks after starting clarithromycin. Remission was defined as a CDAI score of 150 or less, and clinical improvement was defined as a decrease in the CDAI score of at least 100 points.[22]

Statistical analysis

Unless otherwise stated, all numerical data are expressed as the mean ± standard error. The differences of the characteristics between groups were analyzed by the Student's *t*-test, Mann–Whitney *U*-test or Fisher's exact probability test. The evaluation of change of the CDAI score was analyzed by the Wilcoxon signed-rank test.

Results

Clinical response and remission

At the 4-week evaluation, eight patients (57.1%) showed clinical improvement, and six patients (42.9%) showed no clinical response (Fig. 1). The mean CDAI score of all patients decreased significantly from 343.5 (range 164.9–529.2) to 199.8 (range 39.8–376.1) ($P < 0.01$).

At 4 weeks, five (35.7%) of the eight patients with clinical improvement had achieved remission. The mean CDAI score of the eight patients decreased from 349.5 (range 195.4–505.0) to 131.0 (range 39.8–262.2) at 4 weeks. The patient receiving corticosteroids also showed clinical improvement and was tapered off corticosteroids by 4 weeks.

Eight patients with clinical improvement within 4 weeks continued clarithromycin therapy, and six (42.9%) of the eight remained in clinical remission at 12 weeks. We classified the six patients as the responder group. The other two patients (14.3%) relapsed at approximately 6 weeks after starting clarithromycin therapy. In these patients, clarithromycin therapy was stopped, and alternative therapy was started.

Six patients (42.9%) without clinical response at 4 weeks stopped clarithromycin. The mean CDAI score of the six patients at entry and at 4 weeks was 335.6 (range 164.9–529.2) and 291.7

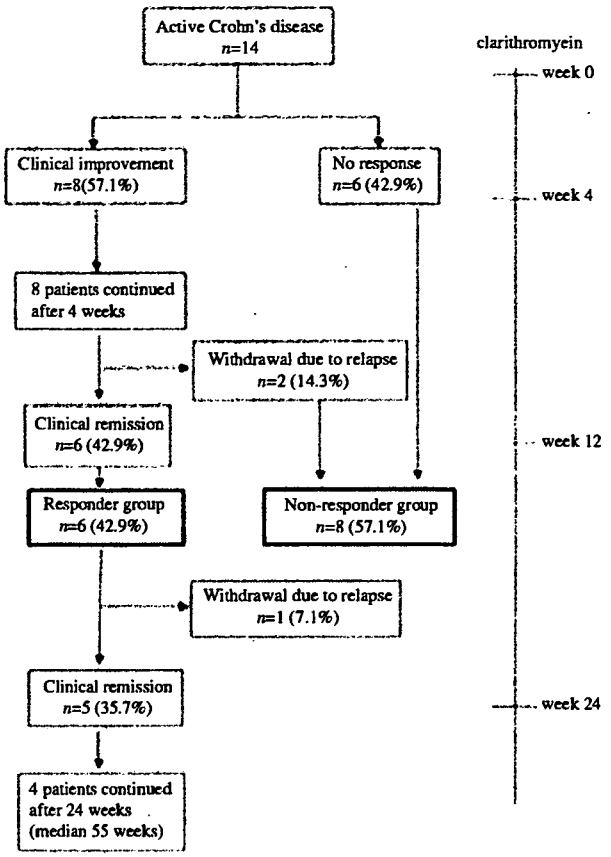

Figure 1 Clinical response in patients with active Crohn's disease receiving clarithromycin ($n = 14$). Clinical activity was evaluated by the Crohn's Disease Activity Index (CDAI) at entry, and at 4, 12, and 24 weeks after starting clarithromycin.

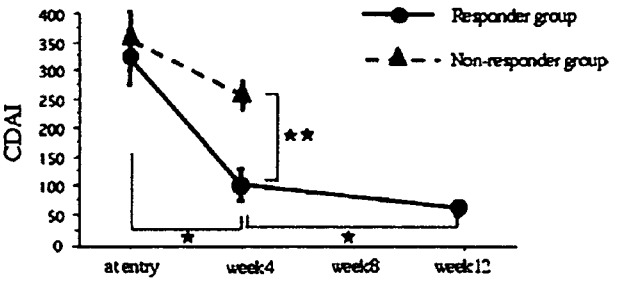

Figure 2 Change in the Crohn's Disease Activity Index (CDAI) score at entry, and at 4 and 12 weeks after starting clarithromycin in responder ($n = 6$) and non-responder ($n = 8$) groups. $^{*}P < 0.05$; $^{**}P < 0.01$.

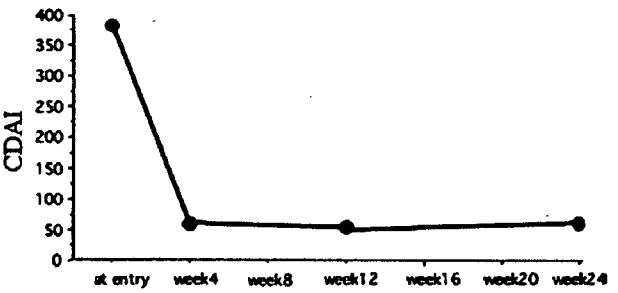

Figure 3 Change in the Crohn's Disease Activity Index (CDAI) score at entry, and at 4, 12, and 24 weeks after starting clarithromycin in one responder. In spite of treatment with azathioprine, his CDAI score was high at entry. After he started clarithromycin therapy, he achieved remission within 4 weeks, and stayed in remission for 24 weeks.

(range 232.4–376.1), respectively. We classified these six patients and the two patients who relapsed 6 weeks after starting clarithromycin as the non-responder group. The mean CDAI score of the non-responder group was 358.9 ± 47.2 (entry), and 272.2 ± 23.2 (4 weeks) (Fig. 2).

All of the responders continued clarithromycin therapy for more than 12 weeks. One of them relapsed at 20 weeks. He was treated with oral tacrolimus and infliximab therapy to achieve remission. The mean CDAI score of the responder group was 323.1 ± 46.9 at entry, 103.4 ± 26.9 at 4 weeks, and 71.2 ± 12.7 at 12 weeks (Fig. 2). Four patients (28.6%) continued clarithromycin therapy after 24 weeks, and all remained in remission.

An effective case

A 35-year-old man had had ileocolonic type Crohn's disease for 17 years. He had undergone intestinal resection twice due to vesico-rectal fistula and severe intestinal stenosis. He was started on azathioprine 16 months prior to our clarithromycin trial, but he often showed symptoms such as abdominal pain and distention. After he was started on clarithromycin therapy, his symptoms soon disappeared and he achieved remission within 4 weeks. His CDAI score during clarithromycin therapy was 384.1 at entry, 62.1 at 4 weeks, 52.5 at 12 weeks, and 62.1 at 24 weeks (Fig. 3). No side-effects were observed in this patient.

Concomitant therapy

In the responder group, three (50%) of six patients were receiving azathioprine, whereas only one of eight patients (12.5%) in the non-responder group was receiving azathioprine ($P = 0.24$) (Table 3). There were no differences in the numbers of patients who were receiving 5-aminosalicylate or sulfasalazine and elemental diet therapy between the responder and non-responder groups.

Relationship between responsiveness to clarithromycin and C-reactive protein levels or disease type

The mean serum C-reactive protein levels at entry of the non-responder group tended to be higher compared with the responder group (35.4 mg/L *vs* 16.3 mg/L, $P = 0.24$), although the difference was not statistically significant (Table 3). There was no difference in age, sex, duration of disease, disease location, intestinal complication, previous intestinal resection, or CDAI score at entry between the two groups (Table 3).

Table 3 Characteristics of responder and non-responder groups

Characteristic	Responder group ($n = 6$)	Non-responder group ($n = 8$)
Age (years)		
Mean	37.0 ± 3.5	31 1 ± 1.6
Range	26–48	27–38
Sex		
Male	5 (83.3)	5 (62.5)
Female	1 (16.7)	3 (37.5)
Duration of disease (years)		
Mean	13.2 ± 4.0	12.0 ± 2.3
Range	1–25	1–20
Disease location		
Colonic	1 (16.7)	0 (0)
Ileocolonic	5 (83.3)	7 (87.5)
Small bowel only	0 (0)	1 (12.5)
Intestinal complication		
Stenosis	2 (33.3)	3 (37.5)
Abscess	1 (16.7)	1 (12.5)
Fistula	2 (33.3)	1 (12.5)
Previous resection	1 (16.7)	3 (37.5)
Concomitant therapy		
Elemental diet	5 (83.3)	6 (75.0)
5-Aminosalicylate, SASP	4 (66.7)	7 (87.5)
Steroids	1 (16.7)	0 (0)
Azathioprine	3 (50)	1 (12.5)
CDAI score		
Entry	323.1 ± 46.9	358.9 ± 47.2
4 weeks	103.4 ± 26.9	272.2 ± 23.2
12 weeks	71.2 ± 12.7	-
C-reactive protein (mg/L)		
Entry	16.3 ± 3.0	35.4 ± 12.9
4 weeks	7.3 ± 2.9	18.5 ± 13.2
12 weeks	9.7 ± 3.8	-

Values shown as *n* (%) per group, or as mean ± SE. CDAI, Crohn's Disease Activity Index; SASP, sulfasalazine.

Side-effects

A minor side-effect was observed in only one patient (7.1%). He withdrew due to abdominal fullness, which improved after stopping clarithromycin. Patients on clarithromycin therapy for more than 24 weeks showed no side-effects.

Discussion

To our knowledge, this is the first demonstration of a therapeutic effect of clarithromycin in Japanese patients with Crohn's disease. A significant clinical response was observed in eight (57.1%) of 14 patients after 4 weeks, and remission was achieved in six patients (42.9%) at 12 weeks. Four patients (28.6%) continued the clarithromycin therapy for more than 24 weeks, with no serious side-effects. These results suggest that clarithromycin is effective for a subpopulation of patients with active Crohn's disease.

In a recently published open label study by Leiper *et al.*, 25 patients with active Crohn's disease received clarithromycin for 4 weeks continuing for up to 12 weeks.[20] Fifteen patients (60%) and nine patients (36%) were receiving corticosteroids and azathioprine, respectively. In that study, 16 patients (64%) showed clinical improvement, and 12 (48%) achieved remission within 4 weeks. At 12 weeks, 15 (60%) showed clinical improvement, and 11 (44%) remained in remission. This open label study demonstrated a good response to clarithromycin in patients with active Crohn's disease who had been resistant to other conventional therapy. Clarithromycin was also tested in patients with Crohn's disease in combination with ethambutol in a 3-month randomized, placebo-controlled study with a 1-year follow up.[17] Five of 15 treated patients (33%) had active Crohn's disease, and they were receiving corticosteroids, and none received immunosuppressive agents. The results of combination therapy with clarithromycin and ethambutol showed no apparent effect. Therefore, the effect of clarithromycin therapy on patients with Crohn's disease is inconclusive.

Large placebo-controlled studies of Crohn's disease have shown that within 3 months 26–42% of patients with active disease went into remission spontaneously.[23,24] However, after 1 year, only 15–18% of the patients were still in remission. Considering this natural history, the remission rate (42.9%) of our clinical trial at 12 weeks with clarithromycin therapy does not appear so high. However, about 80% of the patients remained in remission for 24 weeks after starting clarithromycin. These results suggested that 400 mg/day of clarithromycin therapy might be effective as maintenance rather than induction therapy of remission. In the future, to clarify this issue, we need to perform a dose escalation study with clarithromycin to investigate how much dose of clarithromycin is optimal for inducing remission of patients with active Crohn's disease.

There are several reports that antibiotic therapy is more effective in patients with colonic involvement than in those with small bowel disease alone.[4,25] In a study of combination therapy with rifabutin and clarithromycin or azithromycin, patients with involvement of both the small and large intestine achieved a better clinical response than patients with small bowel disease alone.[14] In contrast, combination therapy with clarithromycin, rifabutin, and clofazimine showed greater benefit in patients with small bowel disease alone. However, in the open label study of clarithromycin by Leiper *et al.*,[20] subgroup analysis by disease location revealed no significant difference between groups. In our study, we could not analyze the association between the effect of clarithromycin and disease location or phenotype, because of the limited number of patients. Therefore, further investigation in a greater number of patients is needed to clarify the clinical factors, such as disease location and phenotype, that influence effectiveness of clarithromycin therapy in patients with Crohn's disease.

This study revealed that the effectiveness of clarithromycin in patients with Crohn's disease was almost equal to that of single therapy with metronidazole or ciprofloxacin. Moreover, there were only a few minor side-effects of clarithromycin therapy unlike metronidazole and ciprofloxacin. Our data also showed that continuous clarithromycin therapy significantly reduced the mean CDAI score of responders at 12 weeks compared to that at 4 weeks. Thus, another advantage of clarithromycin might be the induction of immunomodulatory functions by its long-term administration, which are independent of its antibacterial activity. Clarithromycin is rapidly taken up by immune cells, which results

in a higher concentration in macrophages and polymorphonuclear cells with levels more than 30-fold the plasma concentration. Clarithromycin suppresses TNF-α, IL-1α, IL-1β, and granulocyte-macrophage colony-stimulating factor, while it increases the synthesis of IL-10 in macrophages.[12] In addition, it also enhances phagocytic function of macrophages.[9] Interestingly, in our study three (50%) of six patients in the responder group were receiving azathioprine, while only one (12.5%) of eight in the non-responder group was receiving azathioprine. The reason why combination therapy with clarithromycin and azathioprine tended to be more effective than azathioprine alone might be that clarithromycin modulates macrophage function, which is impaired in patients with Crohn's disease, in addition to azathioprine-induced lymphocyte suppression.

In conclusion, our uncontrolled trial of clarithromycin therapy in Japanese patients with Crohn's disease shows promising results. This study suggests that the administration of clarithromycin is a therapeutic option for Japanese patients with active Crohn's disease.

Acknowledgments

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GASTROENTEROLOGY

Open label trial of clarithromycin therapy in Japanese patients with Crohn's disease

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Key words
antibiotics, clarithromycin, Crohn's disease, immunomodulator, luminal bacteria

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Abstract

Background and Aim: The pathogenesis of Crohn's disease is unclear, but many studies suggest that luminal bacteria play an important role in chronic intestinal inflammation in patients with this condition. Clarithromycin is a macrolide antibiotic with immunomodulatory activity. The aim of this study was to evaluate the effect of clarithromycin therapy in Japanese patients with Crohn's disease.

Methods: Fourteen patients with active Crohn's disease (12 with ileocolonic, one with colonic, one with small bowel type) were treated with oral clarithromycin 200 mg twice daily for 4 weeks. Patients who showed a clinical response within 4 weeks continued the therapy for up to 24 weeks. Four patients also received azathioprine. Clinical activity was assessed with the Crohn's Disease Activity Index (CDAI) at entry and at 4, 12, and 24 weeks after starting clarithromycin.

Results: The mean CDAI score at entry was 343.5. Within 4 weeks, eight (57.1%) of the 14 patients showed clinical improvement, and five (35.7%) of the eight patients achieved remission. All of those eight patients continued clarithromycin therapy after 4 weeks, and six (42.9%) were in clinical remission at 12 weeks. Of the 14 total patients, four (28.6%) continued clarithromycin for more than 24 weeks, and have remained in remission. Patients who received azathioprine concomitantly had a better response to clarithromycin therapy. No severe side-effects were observed during the study period.

Conclusions: This open label study showed encouraging results of clarithromycin therapy in Japanese patients with active Crohn's disease.

Introduction

The etiology of Crohn's disease remains unclear, and both genetic and environmental factors seem to be involved in its pathogenesis. Recent data suggest that indigenous bacterial flora play an important role in the initiation and perpetuation of chronic intestinal inflammation in patients with Crohn's disease.[1] Therefore, antibiotics are considered as one of the basic therapies.[2] Generally, metronidazole and ciprofloxacin are the antibiotics most often used to treat mild to moderate luminal or fistulizing Crohn's disease.[3] In a double blind, placebo-controlled trial of metronidazole in Crohn's disease, there was a significant reduction in Crohn's Disease Activity Index (CDAI) scores and C-reactive protein levels in the metronidazole-treated group compared with placebo.[4] A controlled 6-month trial of ciprofloxacin in Crohn's disease demonstrated a significant reduction in CDAI scores in the ciprofloxacin-treated group.[5] In combination therapy with metronidazole and ciprofloxacin for 10 weeks, 55 of 72 patients (76%) showed a clinical response and 49 of 72 patients (68%) achieved clinical remission.[6] This combination therapy was also effective for Japanese patients with Crohn's disease.[7]

Clarithromycin is a broad-spectrum macrolide antibiotic. Macrolides, such as clarithromycin and azithromycin, bind to the 50S subunit of the 70S bacterial ribosome, and thereby inhibit bacterial protein synthesis. They accumulate in extremely high levels within macrophages and have prolonged intracellular half-lives.[8] These traits enhance their efficacy against intracellular organisms. Similar to other macrolides, clarithromycin acts not only as an antibiotic but also as an immunomodulator, which can enhance macrophage proliferation, phagocytosis, chemotaxis, and cytocidal activity.[9] Clarithromycin suppresses nuclear factor (NF)-κB activation in response to tumor necrosis factor (TNF)-α in monocytes and lymphocytes,[10] and thus represses production of cytokines such as interleukin (IL)-8, TNF-α, and IL-1.[11–13]

There are several reports of open label studies of treatment for Crohn's disease with clarithromycin,[14–20] although its therapeutic results are still inconclusive. The aim of our trial is to evaluate the

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effectiveness of clarithromycin in Japanese patients with active Crohn's disease.

Methods

Patients

Fourteen patients (10 male, 4 female) with active Crohn's disease (CDAI ≥ 150 for more than 3 weeks) were enrolled in this study. The diagnosis of Crohn's disease was confirmed by clinical, radiological, endoscopic, and histological findings. The study was approved by the ethics committee of Kyoto University Hospital. Informed consent was obtained from all patients. The baseline characteristics of the patients are shown in Tables 1 and 2. All the patients were older than 20 years of age (mean 33.6, range 26–48 years), with ileocolonic ($n = 12$), colonic ($n = 1$), or small bowel-type ($n = 1$) Crohn's disease. At entry, the mean CDAI score was 343.5 (range 164.9–529.2).

Four patients (28.6%) were receiving azathioprine. The dose of azathioprine had been unchanged for more than 3 months before starting clarithromycin. Only one patient (7.1%) was receiving corticosteroids (7.5 mg/day) for 6 weeks prior to clarithromycin therapy. Eleven patients (78.6%) were receiving 5-aminosalicylate or sulfasalazine, and 11 patients (78.6%) were receiving elemental diet therapy. These drugs and therapy were kept unchanged during this study except in the one patient receiving steroid therapy in whom the dose was reduced as he clinically improved.

Table 1 Baseline characteristics of 14 patients with Crohn's disease

Characteristic	*n* (%)
Sex	
Male	10 (71.4)
Female	4 (28.6)
Disease location	
Colonic	1 (7.1)
Ileocolonic	12 (85.7)
Small bowel only	1 (7.1)
Previous resection	4 (28.6)
Treatment	
Elemental diet	11 (78.6)
5-Aminosalicylate, SASP	11 (78.6)
Steroids	1 (7.1)
Azathioprine	4 (28.6)
Intestinal complication	
Stenosis	5 (35.7)
Abscess	2 (14.3)
Fistula	3 (21.4)

SASP, sulfasalazine.

Table 2 Mean characteristics at entry of 14 patients with Crohn's disease

Characteristic	Mean	Median	Range
Age (years)	33.6	32	26–48
Duration of disease (years)	12.5	11.5	1–25
Crohn's Disease Activity Index	343.5	361.9	164.9–529.2
C-reactive protein (mg/L)	27.2	16	1–94

Study design

Patients received clarithromycin 200 mg twice daily for at least 4 weeks while they continued baseline treatment. There have been several reports that long-term administration with 400 mg/day of clarithromycin was well tolerated, safe, and effective for Japanese patients with chronic bronchitis or sinusitis.[21] Therefore, we used the same dose in our clinical trial.

Four weeks after starting clarithromycin therapy, we evaluated the CDAI, and decided whether or not clarithromycin therapy should be continued. The responders continued clarithromycin therapy for 24 weeks. If patients showed no clinical response or relapsed, clarithromycin therapy was stopped and alternative treatment was started. In this study, four patients continued on clarithromycin therapy for a median of 55 weeks (range 36–76) to study the long-term outcomes and side-effects.

Clinical activity was assessed with the CDAI and C-reactive protein levels at entry, and at 4, 12, and 24 weeks after starting clarithromycin. Remission was defined as a CDAI score of 150 or less, and clinical improvement was defined as a decrease in the CDAI score of at least 100 points.[22]

Statistical analysis

Unless otherwise stated, all numerical data are expressed as the mean ± standard error. The differences of the characteristics between groups were analyzed by the Student's *t*-test, Mann–Whitney *U*-test or Fisher's exact probability test. The evaluation of change of the CDAI score was analyzed by the Wilcoxon signed-rank test.

Results

Clinical response and remission

At the 4-week evaluation, eight patients (57.1%) showed clinical improvement, and six patients (42.9%) showed no clinical response (Fig. 1). The mean CDAI score of all patients decreased significantly from 343.5 (range 164.9–529.2) to 199.8 (range 39.8–376.1) ($P < 0.01$).

At 4 weeks, five (35.7%) of the eight patients with clinical improvement had achieved remission. The mean CDAI score of the eight patients decreased from 349.5 (range 195.4–505.0) to 131.0 (range 39.8–262.2) at 4 weeks. The patient receiving corticosteroids also showed clinical improvement and was tapered off corticosteroids by 4 weeks.

Eight patients with clinical improvement within 4 weeks continued clarithromycin therapy, and six (42.9%) of the eight remained in clinical remission at 12 weeks. We classified the six patients as the responder group. The other two patients (14.3%) relapsed at approximately 6 weeks after starting clarithromycin therapy. In these patients, clarithromycin therapy was stopped, and alternative therapy was started.

Six patients (42.9%) without clinical response at 4 weeks stopped clarithromycin. The mean CDAI score of the six patients at entry and at 4 weeks was 335.6 (range 164.9–529.2) and 291.7

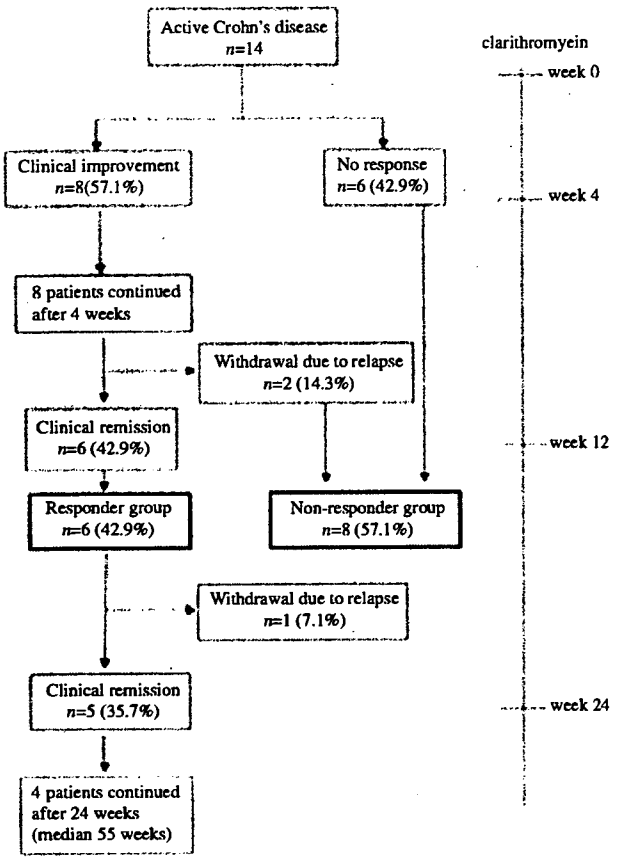

Figure 1 Clinical response in patients with active Crohn's disease receiving clarithromycin ($n = 14$). Clinical activity was evaluated by the Crohn's Disease Activity Index (CDAI) at entry, and at 4, 12, and 24 weeks after starting clarithromycin.

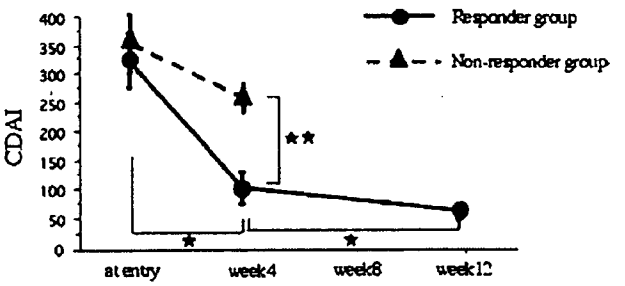

Figure 2 Change in the Crohn's Disease Activity Index (CDAI) score at entry, and at 4 and 12 weeks after starting clarithromycin in responder ($n = 6$) and non-responder ($n = 8$) groups. $^{*}P < 0.05$; $^{**}P < 0.01$.

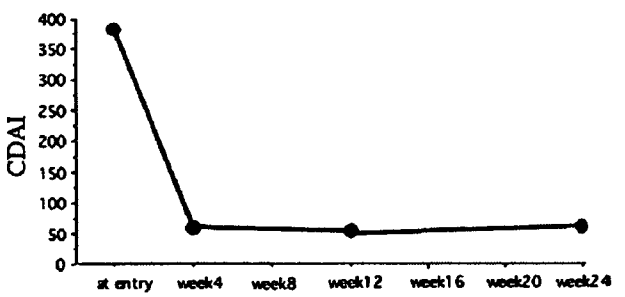

Figure 3 Change in the Crohn's Disease Activity Index (CDAI) score at entry, and at 4, 12, and 24 weeks after starting clarithromycin in one responder. In spite of treatment with azathioprine, his CDAI score was high at entry. After he started clarithromycin therapy, he achieved remission within 4 weeks, and stayed in remission for 24 weeks.

(range 232.4–376.1), respectively. We classified these six patients and the two patients who relapsed 6 weeks after starting clarithromycin as the non-responder group. The mean CDAI score of the non-responder group was 358.9 ± 47.2 (entry), and 272.2 ± 23.2 (4 weeks) (Fig. 2).

All of the responders continued clarithromycin therapy for more than 12 weeks. One of them relapsed at 20 weeks. He was treated with oral tacrolimus and infliximab therapy to achieve remission. The mean CDAI score of the responder group was 323.1 ± 46.9 at entry, 103.4 ± 26.9 at 4 weeks, and 71.2 ± 12.7 at 12 weeks (Fig. 2). Four patients (28.6%) continued clarithromycin therapy after 24 weeks, and all remained in remission.

An effective case

A 35-year-old man had had ileocolonic type Crohn's disease for 17 years. He had undergone intestinal resection twice due to vesico-rectal fistula and severe intestinal stenosis. He was started on azathioprine 16 months prior to our clarithromycin trial, but he often showed symptoms such as abdominal pain and distention. After he was started on clarithromycin therapy, his symptoms soon disappeared and he achieved remission within 4 weeks. His CDAI score during clarithromycin therapy was 384.1 at entry, 62.1 at 4 weeks, 52.5 at 12 weeks, and 62.1 at 24 weeks (Fig. 3). No side-effects were observed in this patient.

Concomitant therapy

In the responder group, three (50%) of six patients were receiving azathioprine, whereas only one of eight patients (12.5%) in the non-responder group was receiving azathioprine ($P = 0.24$) (Table 3). There were no differences in the numbers of patients who were receiving 5-aminosalicylate or sulfasalazine and elemental diet therapy between the responder and non-responder groups.

Relationship between responsiveness to clarithromycin and C-reactive protein levels or disease type

The mean serum C-reactive protein levels at entry of the non-responder group tended to be higher compared with the responder group (35.4 mg/L *vs* 16.3 mg/L, $P = 0.24$), although the difference was not statistically significant (Table 3). There was no difference in age, sex, duration of disease, disease location, intestinal complication, previous intestinal resection, or CDAI score at entry between the two groups (Table 3).

Table 3 Characteristics of responder and non-responder groups

Characteristic	Responder group ($n = 6$)	Non-responder group ($n = 8$)
Age (years)		
Mean	37.0 ± 3.5	31.1 ± 1.6
Range	26–48	27–38
Sex		
Male	5 (83.3)	5 (62.5)
Female	1 (16.7)	3 (37.5)
Duration of disease (years)		
Mean	13.2 ± 4.0	12.0 ± 2.3
Range	1–25	1–20
Disease location		
Colonic	1 (16.7)	0 (0)
Ileocolonic	5 (83.3)	7 (87.5)
Small bowel only	0 (0)	1 (12.5)
Intestinal complication		
Stenosis	2 (33.3)	3 (37.5)
Abscess	1 (16.7)	1 (12.5)
Fistula	2 (33.3)	1 (12.5)
Previous resection	1 (16.7)	3 (37.5)
Concomitant therapy		
Elemental diet	5 (83.3)	6 (75.0)
5-Aminosalicylate, SASP	4 (66.7)	7 (87.5)
Steroids	1 (16.7)	0 (0)
Azathioprine	3 (50)	1 (12.5)
CDAI score		
Entry	323.1 ± 46.9	358.9 ± 47.2
4 weeks	103.4 ± 26.9	272.2 ± 23.2
12 weeks	71.2 ± 12.7	–
C-reactive protein (mg/L)		
Entry	16.3 ± 3.0	35.4 ± 12.9
4 weeks	7.3 ± 2.9	18.5 ± 13.2
12 weeks	9.7 ± 3.8	–

Values shown as *n* (%) per group, or as mean ± SE. CDAI, Crohn's Disease Activity Index; SASP, sulfasalazine.

Side-effects

A minor side-effect was observed in only one patient (7.1%). He withdrew due to abdominal fullness, which improved after stopping clarithromycin. Patients on clarithromycin therapy for more than 24 weeks showed no side-effects.

Discussion

To our knowledge, this is the first demonstration of a therapeutic effect of clarithromycin in Japanese patients with Crohn's disease. A significant clinical response was observed in eight (57.1%) of 14 patients after 4 weeks, and remission was achieved in six patients (42.9%) at 12 weeks. Four patients (28.6%) continued the clarithromycin therapy for more than 24 weeks, with no serious side-effects. These results suggest that clarithromycin is effective for a subpopulation of patients with active Crohn's disease.

In a recently published open label study by Leiper *et al.*, 25 patients with active Crohn's disease received clarithromycin for 4 weeks continuing for up to 12 weeks.[20] Fifteen patients (60%) and nine patients (36%) were receiving corticosteroids and azathioprine, respectively. In that study, 16 patients (64%) showed clinical improvement, and 12 (48%) achieved remission within 4 weeks. At 12 weeks, 15 (60%) showed clinical improvement, and 11 (44%) remained in remission. This open label study demonstrated a good response to clarithromycin in patients with active Crohn's disease who had been resistant to other conventional therapy. Clarithromycin was also tested in patients with Crohn's disease in combination with ethambutol in a 3-month randomized, placebo-controlled study with a 1-year follow up.[17] Five of 15 treated patients (33%) had active Crohn's disease, and they were receiving corticosteroids, and none received immunosuppressive agents. The results of combination therapy with clarithromycin and ethambutol showed no apparent effect. Therefore, the effect of clarithromycin therapy on patients with Crohn's disease is inconclusive.

Large placebo-controlled studies of Crohn's disease have shown that within 3 months 26–42% of patients with active disease went into remission spontaneously.[23,24] However, after 1 year, only 15–18% of the patients were still in remission. Considering this natural history, the remission rate (42.9%) of our clinical trial at 12 weeks with clarithromycin therapy does not appear so high. However, about 80% of the patients remained in remission for 24 weeks after starting clarithromycin. These results suggested that 400 mg/day of clarithromycin therapy might be effective as maintenance rather than induction therapy of remission. In the future, to clarify this issue, we need to perform a dose escalation study with clarithromycin to investigate how much dose of clarithromycin is optimal for inducing remission of patients with active Crohn's disease.

There are several reports that antibiotic therapy is more effective in patients with colonic involvement than in those with small bowel disease alone.[4,25] In a study of combination therapy with rifabutin and clarithromycin or azithromycin, patients with involvement of both the small and large intestine achieved a better clinical response than patients with small bowel disease alone.[14] In contrast, combination therapy with clarithromycin, rifabutin, and clofazimine showed greater benefit in patients with small bowel disease alone. However, in the open label study of clarithromycin by Leiper *et al.*,[20] subgroup analysis by disease location revealed no significant difference between groups. In our study, we could not analyze the association between the effect of clarithromycin and disease location or phenotype, because of the limited number of patients. Therefore, further investigation in a greater number of patients is needed to clarify the clinical factors, such as disease location and phenotype, that influence effectiveness of clarithromycin therapy in patients with Crohn's disease.

This study revealed that the effectiveness of clarithromycin in patients with Crohn's disease was almost equal to that of single therapy with metronidazole or ciprofloxacin. Moreover, there were only a few minor side-effects of clarithromycin therapy unlike metronidazole and ciprofloxacin. Our data also showed that continuous clarithromycin therapy significantly reduced the mean CDAI score of responders at 12 weeks compared to that at 4 weeks. Thus, another advantage of clarithromycin might be the induction of immunomodulatory functions by its long-term administration, which are independent of its antibacterial activity. Clarithromycin is rapidly taken up by immune cells, which results

in a higher concentration in macrophages and polymorphonuclear cells with levels more than 30-fold the plasma concentration. Clarithromycin suppresses TNF-α, IL-1α, IL-1β, and granulocyte-macrophage colony-stimulating factor, while it increases the synthesis of IL-10 in macrophages.[12] In addition, it also enhances phagocytic function of macrophages.[9] Interestingly, in our study three (50%) of six patients in the responder group were receiving azathioprine, while only one (12.5%) of eight in the non-responder group was receiving azathioprine. The reason why combination therapy with clarithromycin and azathioprine tended to be more effective than azathioprine alone might be that clarithromycin modulates macrophage function, which is impaired in patients with Crohn's disease, in addition to azathioprine-induced lymphocyte suppression.

In conclusion, our uncontrolled trial of clarithromycin therapy in Japanese patients with Crohn's disease shows promising results. This study suggests that the administration of clarithromycin is a therapeutic option for Japanese patients with active Crohn's disease.

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Expression of the *REG IV* gene in ulcerative colitis

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The *regenerating gene (REG) IV* gene was isolated from a cDNA library of ulcerative colitis (UC) tissues. However, its role in the pathophysiology of UC and subsequent development of colitic cancer is still unclear. We investigated the expression of the *REG IV* gene in UC and colitic cancer tissues and examined whether cytokines or growth factors are responsible for *REG IV* gene expression and whether *REG IV* gene induction affects cell growth and apoptosis in colon cancer cells. The expressions of *REG IV* and *growth factor* genes in UC tissues were analyzed by real time reverse transcription-polymerase chain reaction. The effects of cytokines and growth factors on *REG IV* gene expression were examined in SW403 cells by Northern blot analysis. The effects of *REG IV* gene induction on cell growth and H_2O_2-induced apoptosis were examined in DLD-1 cells by MTT and TUNEL assays, respectively. *REG IV* mRNA was strongly expressed in inflamed epithelium and in dysplasias and cancerous lesions in UC tissues. The level of *REG IV* mRNA expression was correlated with that of *basic fibroblast growth factor* (*bFGF*) as well as *hepatocyte growth factor* (*HGF*) mRNA expression in UC tissues. The *REG IV* gene expression in SW403 colon cancer cells was enhanced by stimulation with transforming growth factor-α, epidermal growth factor, bFGF, and HGF. *REG IV* gene induction promoted cell growth and conferred resistance to H_2O_2-induced apoptosis in DLD-1 cells. The *REG IV* gene is inducible by growth factors and may function as a growth promoting and/or an antiapoptotic factor in the pathophysiology of UC.
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The pathogenesis of ulcerative colitis (UC) is still unclear, but dysregulated immune function appears to be involved in its chronic inflammatory process, resulting in continuous damage of the colonic mucosa.[1–5] To regenerate the injured colonic tissues, growth factors are thought to play very important roles, and indeed, several growth factors are reported to be upregulated in the colonic tissues in UC.[3,6,7] However, few comprehensive studies have examined the expression patterns of various growth factors in UC tissues simultaneously, and the network of and the relationships among growth factors in UC tissues are not fully understood.

Regenerating gene (*REG*) *IV*, the most recently discovered member of the *REG* gene family, was isolated from a cDNA library of UC tissues by Hartupee *et al.*[8] Although the biological function of REG IV protein is still unclear, REG IV protein may play a role in cell growth because other REG family proteins have been shown to act as growth factors in gastrointestinal organs.[9–14] However, it still remains unknown whether the *REG IV* gene is indeed involved in the pathophysiology of UC and whether REG IV protein really functions as a growth factor. Moreover, it has not been examined how *REG IV* gene expression is regulated. In the present study, therefore, in order to elucidate roles for *REG IV* in the pathophysiology of UC, we investigated the relationship between *REG IV* gene expression and clinicopathological factors in patients with UC, and examined the mechanism of *REG IV* gene expression and the cell growth effect of REG IV protein *in vitro*. Furthermore, to clarify the relationship between the *REG IV* gene and other growth factors in UC mucosa, we examined the expression of various *growth factor* genes together with that of *REG IV*.

MATERIALS AND METHODS

Tissue Specimens and Histological Examination

Colon biopsy specimens were obtained by endoscopy from 22 patients with UC (13 men and nine women; mean age 44.7 years, range 19–79 years; mean disease duration 6.3 years, range 0–19 years) and five normal controls (five men; age range 33–38 years) in 2003 and 2004 at Kyoto University

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Graduate School of Medicine. The tissue specimens were used for real time PCR and histological analyses.

A total of seven colitic cancer lesions (location: five rectum, one sigmoid, one descending; histology: four well-differentiated adenocarcinomas, three mucinous adenocarcinomas) were obtained between 1997 and 2000 from specimens surgically resected from four patients (two men and two women; age range 44–58 years; disease duration 11–25 years) at Dokkyo University School of Medicine. The tissue specimens were fixed in 10% formalin solution, embedded in paraffin, and subjected to histological analyses.

This work was done with the approval of the Review Board of Kyoto University Hospital and the Dokkyo University Surgical Pathology Committee, and informed consent was obtained from all patients. The diagnosis of UC was based on established endoscopic and histologic criteria,[15] and the degree of inflammation was evaluated according to Matts' grade[15] throughout the experiments.

Real Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from colonic biopsy samples with Trizol reagent (Gibco BRL, Rockville, MD, USA). To generate cDNA, 5 μg of total RNA was reverse-transcribed using 200 U of SuperScript II reverse-transcriptase (Gibco BRL) and oligo-dT primer (Applied Biosystems, Branchburg, NJ, USA) in a total reaction volume of 20 μl as described previously.[16] TaqMan quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The set of primers and probe for *human REG IV, epidermal growth factor* (*EGF*), *transforming growth factor* (*TGF*)-α, *basic fibroblast growth factor* (*bFGF*), and *hepatocyte growth factor* (*HGF*) were prepared as shown in Table 1. In addition, a set of primers and probe for human *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was synthesized by Applied Biosystems (Foster City, CA, USA).

Each amplification was done in 50 μl of reaction mixture with 50 ng of cDNA, 250 nM each growth factor probe (or 100 nM GAPDH probe), 900 nM each growth factor primer (or 200 nM GAPDH primer), and 1 × TaqMan universal PCR master mixture (Applied Biosystems, Branchburg, NJ, USA). The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 60 s. A template-free negative control was included in all amplifications, and each assay was performed in duplicate. The intensity of the dye fluorescence was determined, and the expression levels of *growth factor* mRNAs were normalized to *GAPDH* mRNA expression levels.

In Situ Hybridization

The 474-bp sequence of human *REG IV* was inserted into the pCRII vector using the TA cloning system (Invitrogen, Grand Island, NY, USA). To generate digoxigenin (DIG)-labeled sense and antisense RNA probes, the plasmid was linearized with *Bam*HI and *Not*I and transcribed, respectively, with T7 or SP6 RNA polymerase in the presence of DIG-UTP using the DIG-RNA labeling kit (Boehringer, Mannheim, Germany).

Tissue sections (4 μm) were deparaffinized, rehydrated, treated with 18 μg/ml proteinase K at 37°C for 10 min, post-fixed in 4% paraformaldehyde, acetylated with acetic anhydride (0.25% v/v) in 0.1 mol/l triethanolamine, and dehydrated in an ethanol series before hybridization. Hybridization was carried out in hybridization buffer containing DIG-labeled RNA probes (1 μg/ml) at 45°C overnight. After hybridization, the sections were treated with ribonuclease and washed in a solution of 2 × standard saline citrate/50% (v/v) deionized formamide at 55°C for 30 min. Finally, the sections were reacted with anti-DIG antibody, and the signals were visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Roche, Mannheim, Germany).

Table 1 Primers and probes for *REG IV* and *growth factors* used in this study

Human REG IV	5′-TGCACGACCCACAGAAGAG-3′ (sense)
	5′-GACTTGCCAGACCAGGATCT-3′ (antisense)
	5′-FAM-AATCCACTGCCACTGCTG (probe)
Human EGF	5′-CCTGTAACACACATGCAGTGAGA-3′ (sense)
	5′-GGACTGACTTGGAAGGCACTT-3′ (antisense)
	5′-FAM-CTAGGGAGGCGTATAT (probe)
Human TGF-α	5′-ACTGCACGTGCCCTGTAG-3′ (sense)
	5′-ACAGGAAACAAGTTGATGACATCGT-3′ (antisense)
	5′-FAM-ATCAGGAAGCAGAACAAA (probe)
Human bFGF	5′-CCGACGGCCGAGTTGA-3′ (sense)
	5′-CAACTCCTCTCTCTTCTGCTTGAA-3′ (antisense)
	5′-FAM-CCCTCACATCAAGCTAC (probe)
Human HGF	5′-CACCACACCGGCACAAATTC-3′ (sense)
	5′-GGGATTGCGGCAATAATTATCATCA-3′ (antisense)
	5′-FAM-TTGCCTGAAAGATATCC (probe)

Immunohistochemistry

Immunohistochemical staining for REG IV, chromogranin A and Ki67 was performed with an Envision Kit (DAKO, Kyoto, Japan) as described previously,[16] using anti-human REG IV antibody (1:50; R&D Systems, Minneapolis, MN, USA), anti-human chromogranin A antibody (ready to use; Immunotech, Marseille, France) and anti-human Ki67 antibody (1:50; DAKO, Kyoto, Japan). Finally, the sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride with

0.05% H_2O_2 for 5 min and then counterstained with Mayer's hematoxylin.

Effects of Cytokines and Growth Factors on *REG IV* Gene Expression in Colon Cancer Cells

The human colon cancer cell line SW403 was cultured in RPMI1640 medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) in a humidified incubator at 37°C with an atmosphere of 5% CO_2. The cells were seeded in 10-cm dishes (Iwaki, Funabashi, Japan) and used for cytokine and growth factor stimulation tests when the cells reached subconfluence. The cells were stimulated with the indicated amount of TNF-α (Roche, Indianapolis, IN, USA), interleukin (IL)-6 (Roche), IL-8 (Roche), IFN-γ (Roche), IL-1β (Roche), TGF-α (PeproTech Inc., Rocky Hill, NJ, USA), EGF (Roche), bFGF (Sigma, Saint Louis, MO, USA), and HGF (Sigma) in serum-free medium for 12 h. Furthermore, in order to assess the inhibitory effect of MEK inhibitor on *REG IV* gene expression, the cells were pre-incubated with PD98059 (Sigma; 10 and 50 μM) for 1 h, followed by additional incubation with or without bFGF or HGF for the indicated periods. After these treatments, the cells were subjected to Northern and Western blot analyses.

RNA Preparation and Northern Blotting

Total RNA was extracted from each cell line using Trizol reagent (Gibco BRL). Extracted RNA (20 μg) from the colon cancer cell line was separated by electrophoresis in 0.66 M formaldehyde/1% agarose gel. After transfer to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), the nucleic acids were fixed to the membrane by UV cross-linking. The probes used for Northern blot analysis were a 0.47-kilobase (kb) cDNA of human *REG IV* and a 0.63-kb cDNA of human *GAPDH*.[16] The radiolabeling of the probes, hybridization, and detection of signals were performed as described previously.[10]

Western Blotting

Cells were lysed in 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 50 mM NaF, and 1 × proteinase inhibitor (Complete Mini; Roche). Protein extract (20 μg) was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-ERK and anti-phospho-specific ERK antibodies (Cell Signaling, Beverly, MA, USA) at 4°C over night, and then incubated with peroxidase-conjugated secondary anitibodies for 1 h at 37°C. Proteins were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Transfection and Expression of the Human *REG IV* cDNA

The full-length human *REG IV* cDNA with an *Eco*RI site at both the 5′ and 3′ ends was generated by reverse-transcription polymerase chain reaction from human stomach mRNA. The fragment was once ligated in the pCRII vector and cloned. The cloned nucleotides were confirmed by sequencing, excised from the vector by cutting with *Eco*R I, and then inserted into the *Eco*R I restriction site in the pIRES2-EGFP vector containing the cytomegalovirus promoter driving the enhanced green fluorescent protein (EGFP) gene (Clontech, Palo Alto, CA, USA). After cloning and verifying the nucleotides of the human *REG IV* cDNA by sequencing, the construct was named pIRES2-hREG IV, and the pIRES2-EGFP vector without the insert was used as a control.

The plasmids were stably transfected into human colon cancer cell line DLD-1 using FuGENE6 transfection reagent (Roche) according to the manufacturer's protocol. To select cells with stable expression of pIRES2-hREG IV and pIRES2-EGFP, the cells were cultured over 3–4 weeks in medium that contained G-418 (GIBCO; 1000 μg/ml). Surviving colonies were pooled and maintained in the standard culture medium supplemented with G-418 (1000 μg/ml).

BrdU Cell Proliferation Assay

Cell proliferation was assessed by the BrdU Cell Proliferation Assay (Exalpha Biological Inc., Watertown, MA, USA). DLD-1 cells (1×10^4), stably transfected with the pIRES2-hREG IV (DLD-1-REG IV cells) or pIRES2-EGFP (DLD-1-EGFP cells) vector, were plated in 96-well microplates (Iwaki) and incubated in serum-free DMEM for 24 h. At 18 h prior to assessment, 20 μl of 5-bromo-2′-deoxyuridine (BrdU) was added to each well according to the protocol supplied. The cells were fixed and the DNA was denatured using reagents supplied with the assay kit. The cells were incubated with anti-BrdU peroxidase conjugate, washed and incubated with color development substrate. The plates were read at 450 nm in a spectrophotometer (Molecular Devices Co., Sunnyvale, CA, USA).

Cell Growth Assay

Cell growth was assessed by a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), which consists of (2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt: WST-8 and 1-methoxy-5-methylphenazinium methylsulfate: 1-methoxy-PMS. DLD-1-REG IV cells (1×10^4) and control DLD-1-EGFP cells were plated in 96-well microplates (Iwaki). The cells were incubated in serum-free DMEM for 48 h. After addition of 10 μl of the Cell Counting Kit–8 reagent and a 3-h incubation, the plates were read at 450 nm in a spectrophotometer (Molecular Devices Co.). In another experiment, the cells (5×10^4) were incubated in serum-free DMEM for 24 and 48 h, followed by washing with PBS, and then harvested. The number of viable cells was counted with a Particle Counter Z1 system (Beckman Coulter, Hialeah, FL, USA).

Caspase Assay

Caspase activity was assessed using a Colorimetric CaspACE Assay System (Promega, Madison, WI, USA). DLD-1-REG

IV (1×10^6) and control DLD-1-EGFP cells were cultured in 6-cm culture dishes (Iwaki) and incubated in serum-free medium for 24 h. The cells were then incubated for 2 h with different concentrations (0–0.1 mmol/l) of H_2O_2 in serum-free medium. Thereafter, the cells were incubated in serum-free medium for 24 h and resuspended in lysis buffer. Lysate (50 μl) was reacted with Ac-DEVD-pNA in the 96-well microplate according to the manufacturer's protocol. For measurement of caspase-3 activity, the plates were read at 405 nm in a spectrophotometer (Molecular Devices Co.).

TUNEL Assay

DLD-1-REG IV cells (2×10^4) and control DLD-1-EGFP cells were cultured in 4-well culture slides (Falcon, Bedford, MA, USA). After 24 h, the cells were incubated for 2 h with different concentrations (0–5 mmol/l) of H_2O_2 in serum-free medium. Thereafter, the cells were incubated in the routine medium for 24 h. After washing with PBS, the slides were fixed with 10% buffered formalin for 15 min and then treated with 0.3% H_2O_2 in methanol for 30 min at room temperature. The slides were then subjected to incubation with 0.1% TritonX-100 in 0.1% sodium citrate for 2 min on ice and stained using an *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN, USA) according to the supplied protocol. Briefly, the pretreated slides were incubated in TdT-mediated dUTP nick end-labeling (TUNEL) reaction mixture for 60 min at 37°C. The slides were then washed in PBS, incubated with peroxidase-conjugated Fab fragments of anti-fluorescein at 37°C for 30 min, washed in PBS, and visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB). One hundred cells were counted in five different visual fields (magnification, × 200) on each section. TUNEL index was calculated as the percentage of positive cells.

Cell Survival Assay

DLD-1 cells (4×10^4), stably transfected with pIRES2-hREG IV (DLD-1-REG IV cells) or pIRES2-EGFP (DLD-1-EGFP) vectors, were plated in 12-well microplates (Iwaki). Thereafter, the cells were incubated for 2 h with different concentrations (0–5 mmol/l) of H_2O_2 in serum-free medium. Then, the cells were incubated in the routine medium for 24 h, and the number of surviving cells was evaluated by trypan blue exclusion.

Akt Phosphorylation

To prepare conditioned medium, we cultured human embryonic kidney (HEK) 293 T cells in DMEM medium supplemented with 10% fetal bovine serum. In accordance with the manufacturer's protocol, cells were transfected with 10 μg of pIRES2-hREG IV or control plasmid using Lipofectamine 2000 transfection reagent (Invitrogen). The medium was replaced by serum-free RPMI1640 medium after a 48-h incubation period. The conditioned medium was then collected and stored frozen as a source of recombinant REG IV protein.

DLD-1 cells were cultured in 10-cm dishes for 24 h. After washing with PBS, the medium was changed to conditioned medium containing human recombinant REG IV or control medium, and the cells were incubated for another 12 h. The cells were then mixed with lysis buffer as reported previously.[9] Protein extract (10 μg) was fractionated by sodium dodecyl sulfate polyacrylamide gel electropholesis, transferred to a polyvinylidene difluoride membrane, and Western blotting was performed using anti-Akt, antiphospho-specific Akt (Ser473) (New England Biolabs, Beverly, MA, USA), and anti-β-actin antibodies (Sigma Chemical Co., St Louis, MO, USA), as reported previously.[9]

Statistical Analysis

All values are expressed as the mean ± s.e.m. Significance of differences between two groups was assessed by the unpaired two-tailed *t* test, or by the Mann–Whitney *U*-test when data were not parametric. The relationships among *REG IV* and *growth factor* mRNA levels were assessed by linear regression analysis. A $P<0.05$ was considered to indicate statistical significance.

RESULTS

Detection of *REG IV* Gene Expression in Normal Colonic and Ulcerative Colitis Mucosa, and in Dysplasia and Colitic Cancer by *In Situ* Hybridization

In normal human colonic mucosa, *REG IV* mRNA was detected in only a few epithelial cells in the crypts (Figure 1a). On the other hand, in ulcerative colitis mucosa, the number of *REG IV* mRNA-positive epithelial cells was increased, and the signal intensity in each cell was enhanced (Figure 1b).

REG IV gene expression was detected in both dysplastic and cancerous cells in all seven samples of colitic cancer from the four patients examined, and the signal intensity was apparently stronger than that in normal colonic epithelial cells (Figure 1c and d). No signal was detected when using the DIG-labeled sense probe throughout the experiments (data not shown).

Expression of REG IV Protein, Chromogranin A and Ki67 in Normal Colonic and Ulcerative Colitis Mucosa, and in Dysplasia and Colitic Cancer

In normal colonic mucosa, REG IV and chromogranin A were co-expressed in a few epithelial cells in the basal portion of crypts (Figure 2a and b). However, some epithelial cells alternatively expressed REG IV or chromogranin A (Figure 2a and b). REG IV was also expressed in goblet cells at various intensities (Figure 2b). In UC mucosa, the number of REG IV-positive cells and chromogranin A-positive cells in the crypts was increased (Figure 2d and e). Additionally, we found that REG IV-positive cells were mainly present in the lower part of the colonic mucosa (Figure 2e). REG IV protein was also strongly expressed in the most of the dysplastic and cancerous cells (Figure 2h and k), whereas chromogranin A was hardly expressed in these cells (Figure 2g and j). On the

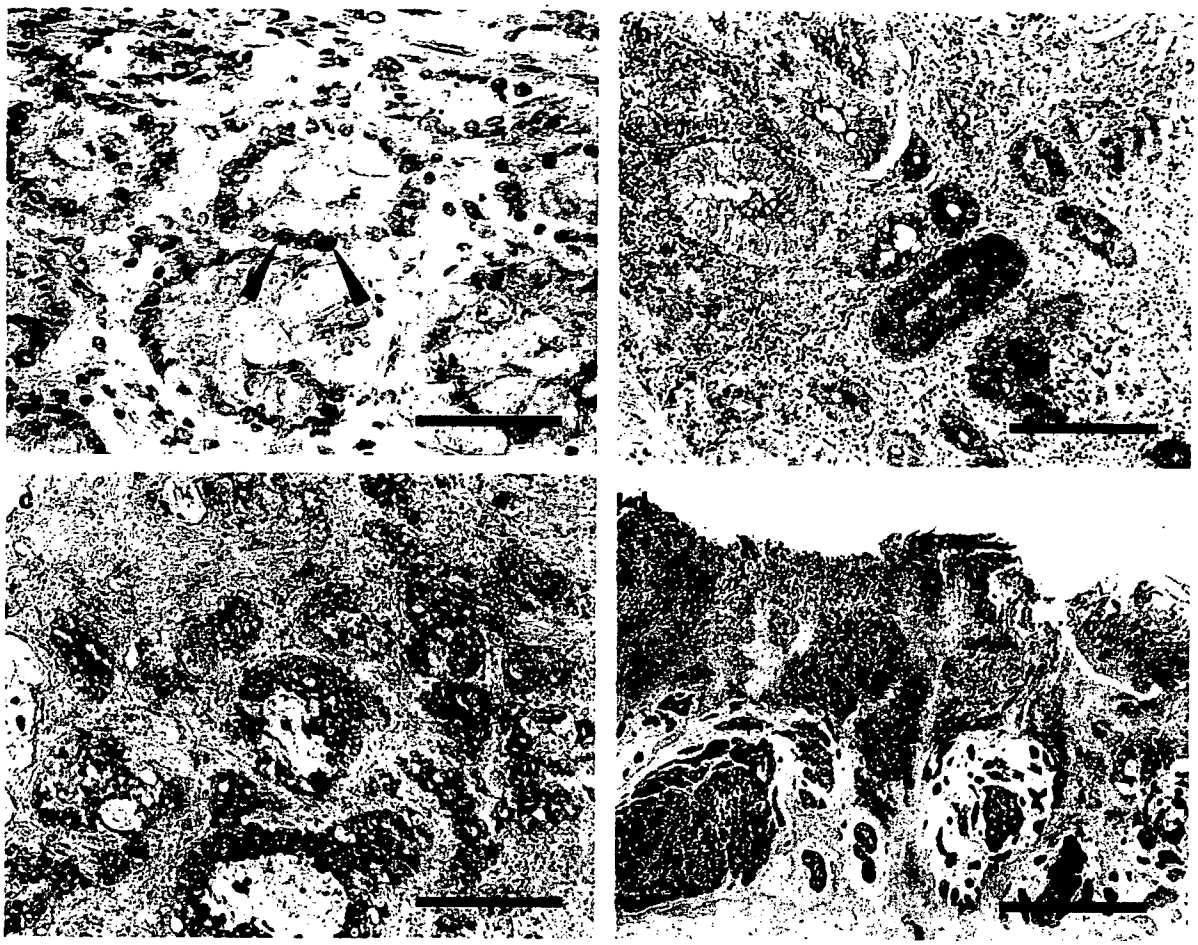

Figure 1 *In situ* hybridization of *REG IV* mRNAs in normal colon (**a**), UC (**b**), dysplasia (**c**), and colitic cancer (**d**) tissues. (**a**) Normal human colonic mucosa. Hybridized signals for *REG IV* mRNA (arrowheads) are evident in only a few epithelial cells in the crypts (bar = 50 μm). Inflamed (**b**) and dysplastic (**c**) epithelial cells in UC mucosa. Both the number of *REG IV* mRNA-positive cells and the signal intensity in each cell are markedly increased (bar = 100 μm). (**d**) Colitic cancer tissue. The nested cancer cells express very strong signals for *REG IV* mRNA (bar = 200 μm). (**a–d**) Tissue sections were visualized with NBT/BCIP and counter-stained with methyl green.

other hand, both the distribution and numerical change of Ki67-positive cells were relatively similar to those of REG IV-positive cells in normal, inflamed, dysplastic and cancerous tissues (Figure 2c, f, i, and l).

Expression of *REG IV* and *Growth Factor* mRNAs in Normal Colonic and Ulcerative Colitis Mucosa

REG IV mRNA expression was detectable by real-time PCR in all samples of colonic mucosa from both control and UC patients. As shown in Figure 3a, the level of *REG IV* mRNA expression was significantly greater in UC tissues than in normal colonic tissues ($P<0.05$). In addition, the levels of *bFGF* and *HGF* mRNA expression were significantly greater in UC tissues than in normal colonic tissues ($P<0.05$), and the *TGF-α* mRNA expression level tended to be increased in UC tissues. We then analyzed the relationship between the severity of inflammation and *REG IV* or *growth factor* mRNA expression (Figure 3b). In the Matts 1, 2 score group, none of the genes examined showed a difference in expression level from those in the control group, although the *REG IV* and *HGF* mRNA expression levels in the Matts 1, 2 score group tended to be higher in UC tissues than in normal colonic tissues. In the Matts 3, 4, 5 score group, *REG IV*, *bFGF* and, *HGF* mRNA expression levels were significantly increased ($P<0.05$ *vs* control). Although there was no significant difference, the *TGF-α* mRNA expression level in the Matts 3, 4, 5 score group tended to be higher in UC tissues than in normal colonic tissues. On the other hand, in none of the genes examined was there any significant relationship between expression level and age, sex, or disease duration (data not shown).

Furthermore, we analyzed the correlation between *REG IV* and growth factor expression in UC tissues. The expression level of *REG IV* was correlated significantly with that of *bFGF* ($P<0.05$) and *HGF* ($P<0.05$) but not with that of *EGF* or *TGF-α* in UC tissues (Figure 3c and d).

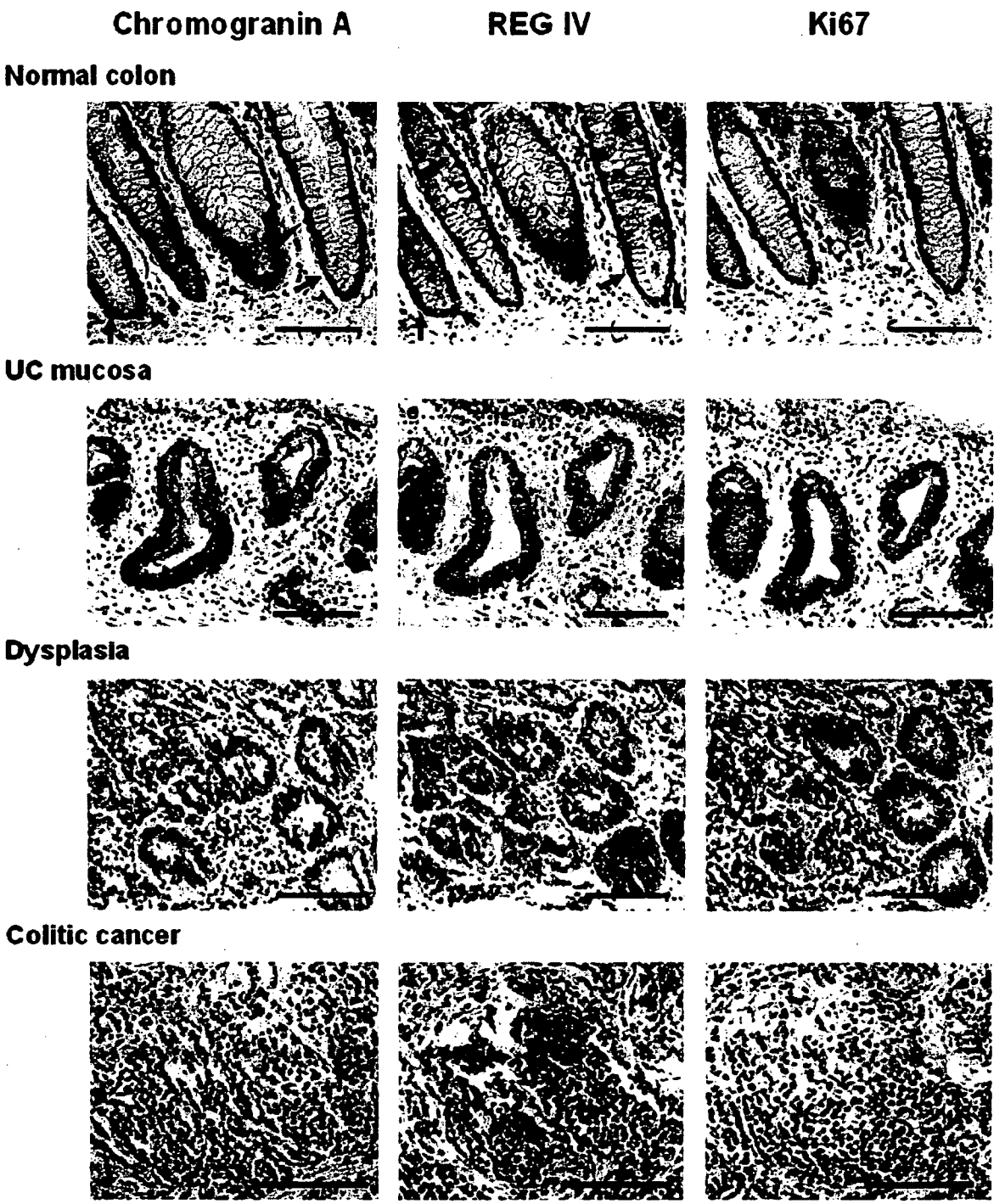

Figure 2 Serial immunostainings of REG IV protein, chromogranin A and Ki67 in normal colonic (**a–c**) and UC mucosa (**d–f**), and in dysplasia (**g–i**) and colitic cancer (**j–l**). In normal colonic mucosa, REG IV and chromognanin A were co-expressed in a few epithelial cells in the basal portion of crypts (**a**, **b**, arrows). However, some epithelial cells alternatively expressed REG IV or chromogranin A (**a**, **b**, arrows heads). In UC mucosa, the number of chromogranin A-positive cells (**d**) and REG IV-positive cells (**e**) in the crypts was increased. REG IV protein was also strongly expressed in the most of the dysplastic (**h**) and cancerous cells (**k**), whereas chromogranin A was hardly expressed in these cells (**g**, **j**). Both the distribution and numerical change of Ki67-positive cells were relatively similar to those of REG IV-positive cells in normal (**c**), inflamed (**f**), dysplastic (**i**) and cancerous tissues (**l**). Bars = 100 μm.