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).

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)

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).

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) 293T 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 $\pm$ 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

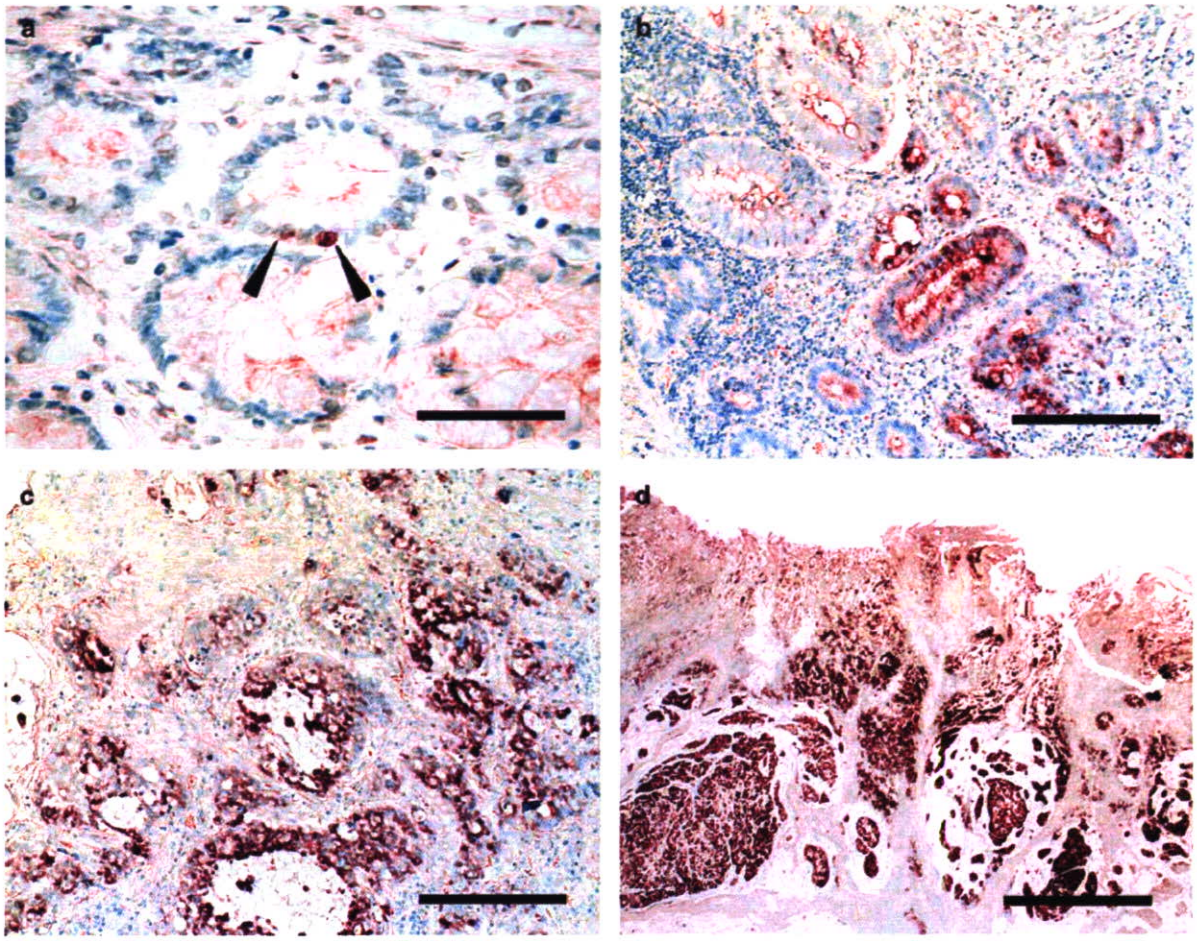

Figure 1 *In situ* hybridization of *REG IV* mRNAs in normal colon (**a**), UC (**b**), dysplasia (**c**), and colitic cancer (**d**) tissues. (**a**) Normal human colonic mucosa. Hybridized signals for *REG IV* mRNA (arrowheads) are evident in only a few epithelial cells in the crypts (bar = 50 μm). 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).

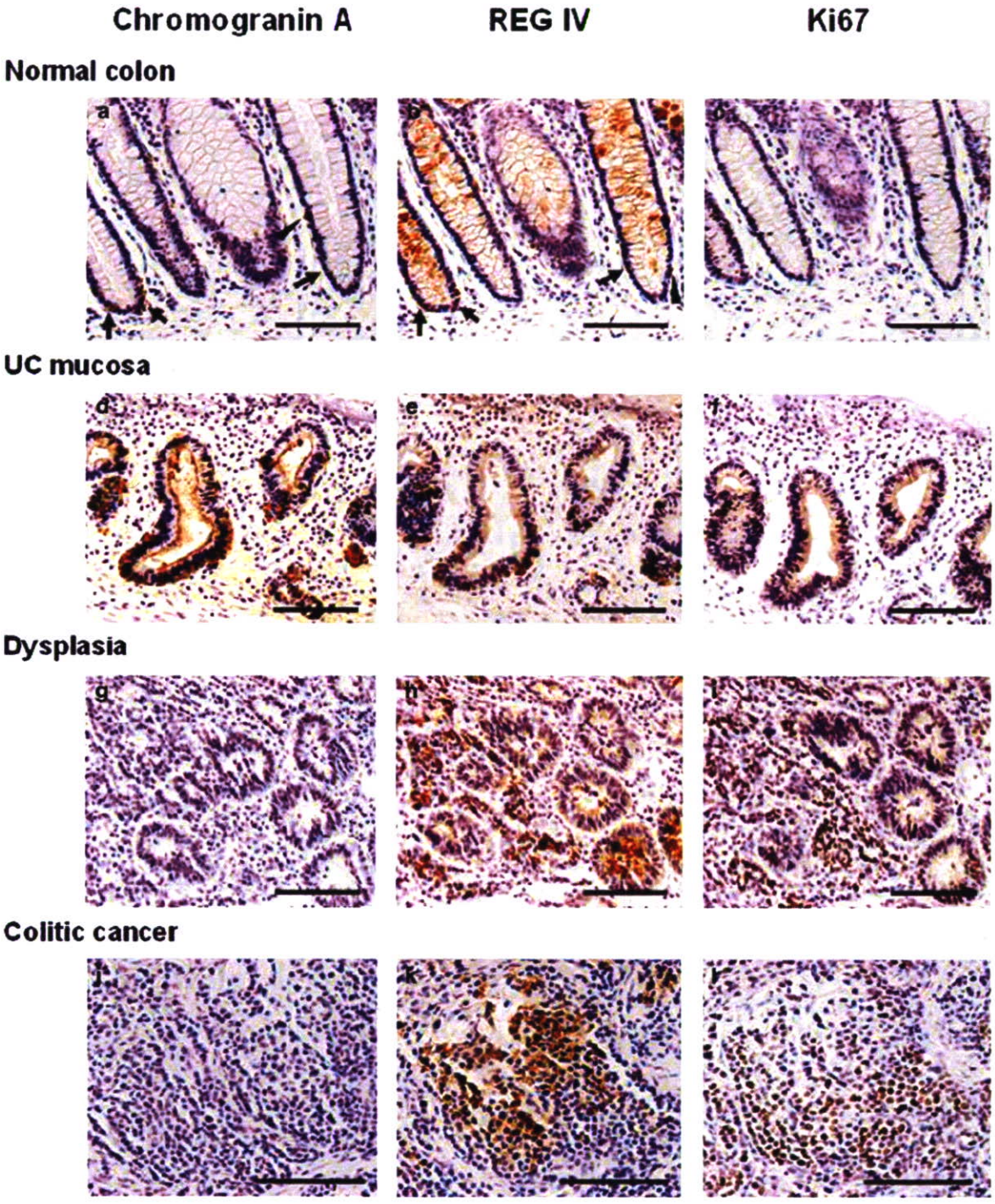

Figure 2 Serial immunostainings of REG IV protein, chromogranin A and Ki67 in normal colonic (**a–c**) and UC mucosa (**d–f**), and in dysplasia (**g–i**) and colitic cancer (**j–l**). In normal colonic mucosa, REG IV and chromognanin A were co-expressed in a few epithelial cells in the basal portion 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.

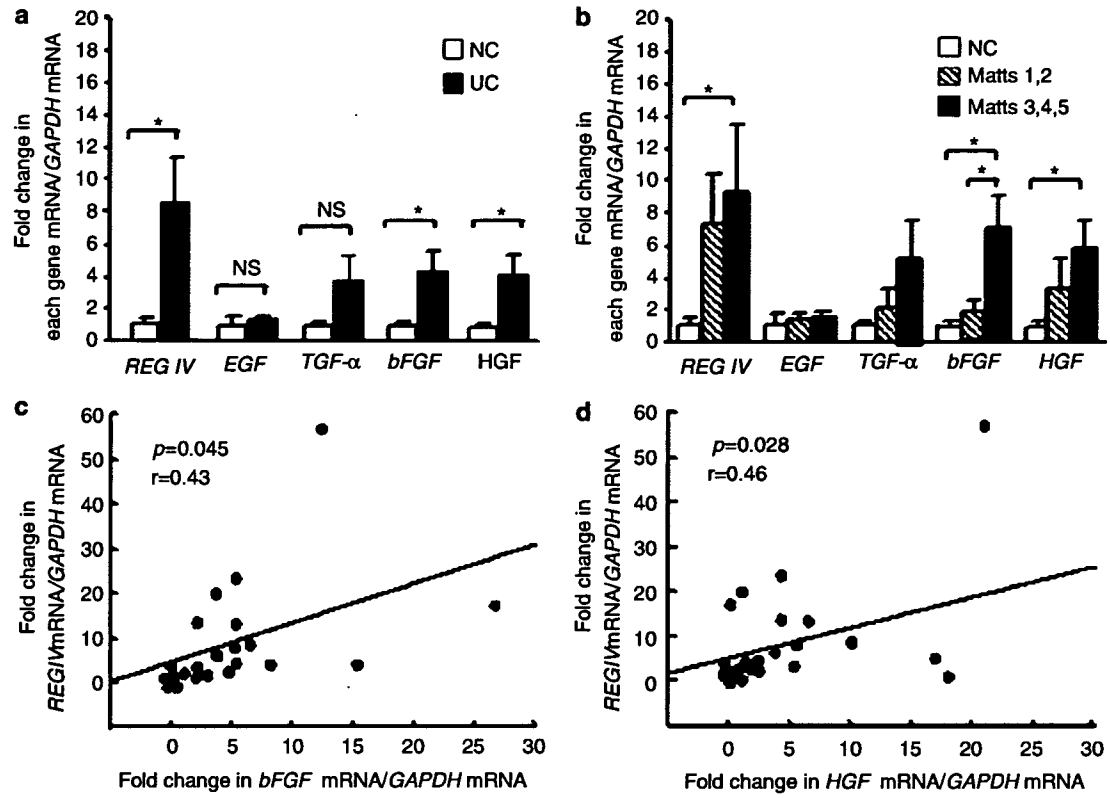

Figure 3 *REG IV* mRNA expression in UC tissues. (**a**) Expression levels of mRNA for *REG IV* and other *growth factors* in normal colon and UC tissues. (**b**) Comparison of mRNA expression levels for *REG IV* and other *growth factors* among UC groups subdivided by histological findings. Correlation between *REG IV* and *bFGF* (**c**) or *HGF* (**d**) gene expression levels in UC tissues. All results are expressed as fold change in *REG IV*, *bFGF*, and *HGF* mRNA/*GAPDH* mRNA ratio relative to the normal control group. Significantly different between two groups: $^*P<0.05$. NS; not significant.

Effects of Cytokines and Growth Factors on *REG IV* Gene Expression in Colon Cancer Cell Line SW403

None of TNF-α (500 and 1000 IU/ml), IL-6 (100 and 1000 IU/ml), IL-8 (1 and 10 ng/ml), IFN-γ (100 and 500 IU/ml) or IL-1β (50 and 500 IU/ml) had any significant effect on *REG IV* gene expression in human colon cancer cell line SW403 (Figure 4). In contrast, treatment with TGF-α (1 and 10 nM), EGF (10 and 100 nM), bFGF (1 and 10 nM) or HGF (0.1 and 1 nM) significantly enhanced *REG IV* mRNA expression in this cell line. Similar results were also obtained from the $CaCO_2$ colon cancer cell line (data not shown).

Effects of bFGF and HGF on *REG IV* Gene Expression in Colon Cancer Cell Line SW403

As mentioned above, treatment with bFGF (10 nM) or HGF (1 nM) significantly enhanced *REG IV* mRNA expression in SW403 cells. The increase of *REG IV* mRNA expression by bFGF as well as by HGF was associated with a significant enhancement of ERK phosphorylation (Figure 5a and b). The enhanced phosphorylation of ERK by bFGF (10 nM) as well as by HGF (1 nM) was suppressed by concomitant administration of the MAPK kinase inhibitor PD98059 (10 and 50 μM) (Figure 5b), and the decreases of ERK phosphorylation by PD98059 were accompanied by a reduction of *REG IV* mRNA expression (Figure 5a and b).

Effects of *REG IV* Gene Induction on BrdU Incorporation and Cell Growth in DLD-1 Cells

DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) showed significantly higher BrdU incorporation than did DLD-1 cells transfected with pIRES2-EGFP (DLD-1-EGFP; control) ($P<0.01$) (Figure 6a). Moreover, DLD-1-REG IV cells showed significantly higher WST-8 cleavage levels than did DLD-1-EGFP cells ($P<0.01$) (Figure 6b), suggesting that *REG IV* gene induction enhances the growth activity of DLD-1 cells. Compatible with these results, *REG IV* gene induction in DLD-1 cells significantly promoted numerical cell growth at 24 and 48 h of culture time (Figure 6c). Similar results were obtained using the HT29 colon cancer cell line (data not shown).

Effects of *REG IV* Gene Induction on Apoptosis and Survival of DLD-1 Cells

We examined both specific caspase-3 activity and TUNEL positivity to compare the apoptotic effects of H_2O_2 treatment on DLD-1 cells transfected with the pIRES2-hREG IV and

pIRES2-EGFP (control) plasmids. As shown in Figure 7a, the caspase activity of DLD-1-REG IV cells in response to H_2O_2 (0.03 and 0.1 mM) was significantly lower than that of DLD-1-EGFP cells. In addition, the DLD-1-REG IV cells showed significantly lower TUNEL positivity than the DLD-1-EGFP cells when they were treated with H_2O_2 at concentrations of 1-5 mM, suggesting that REG IV-overexpressing cells are more resistant to apoptosis induced by H_2O_2 (Figure 7b). Consistent with these data, the DLD-1-REG IV cells showed a significantly higher survival rate than the DLD-1-EGFP cells upon exposure to H_2O_2 (1-5 mM) (Figure 7c).

We then examined the alteration of intracellular signaling by REG IV protein, and found that treatment with REG IV conditioned medium enhanced the phosphorylation of Akt in DLD-1 cells (Figure 7e).

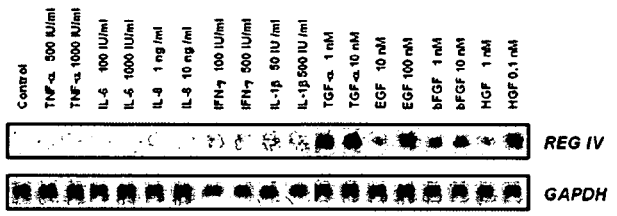

Figure 4 Effects of various cytokines and growth factors on *REG IV* mRNA expression in SW403 cells. Total RNA (20 μg) was extracted 12 h after stimulation with cytokines or growth factors and analyzed by Northern blotting using ^{32}P-labeled cDNAs for *REG IV* and *GAPDH* mRNA.

DISCUSSION

REG IV, a newly characterized member of the regenerating gene family, was isolated from a cDNA library of UC tissues,[8] implying that the *REG IV* gene plays some roles in the pathophysiology of UC. In the present study, we demonstrated histologically that in the normal crypt base REG IV protein is expressed in a few epithelial cells showing neuroendocrine features. This finding, which is similar to that for REG Iα protein in colon tissues,[12] is compatible with the observation

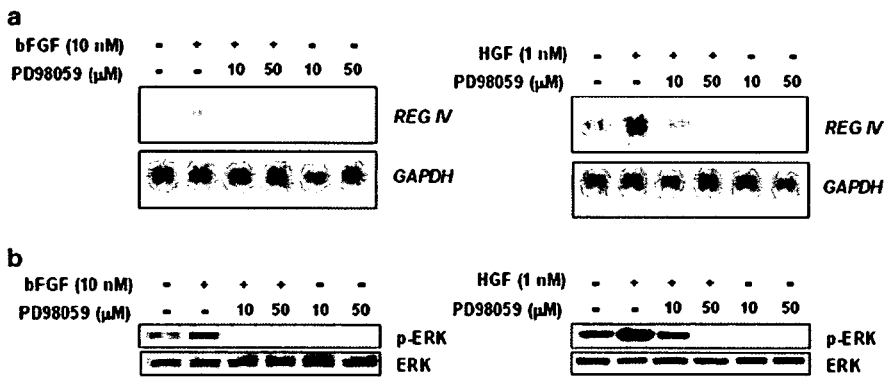

Figure 5 Effects of bFGF and HGF on *REG IV* gene expression via the MAPK pathway in SW403 cells. (**a**) The cells were preincubated with 10 or 50 μM PD98059 for 1 h and stimulated with 10 nM bFGF or 1 nM HGF for 12 h. Total RNA (20 μg) was extracted and analyzed by Northern blotting using ^{32}P-labeled cDNAs for *REG IV* and *GAPDH* mRNA. (**b**) The cells were preincubated with 10 or 50 μM PD98059 for 1 h and stimulated with 10 nM bFGF or 1 nM HGF for 15 min. Cell lysates were prepared and subjected to immunoblot analysis with anti-ERK and anti-phospho-specific ERK antibodies.

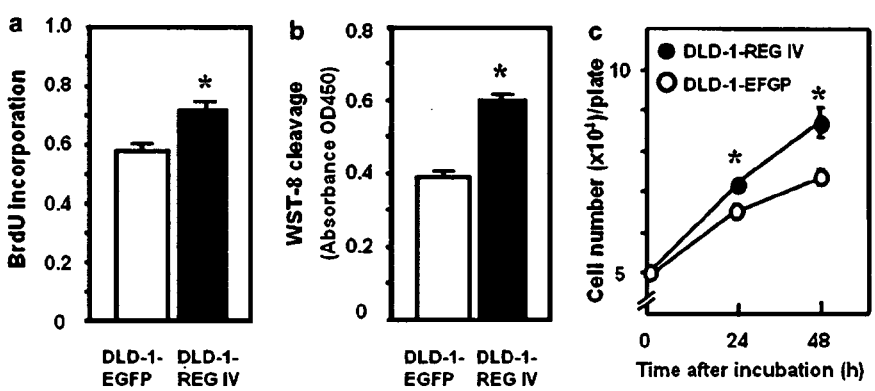

Figure 6 Effects of *REG IV* gene induction on BrdU incorporation by (**a**) and growth of (**b**, **c**) human colon cancer cells. DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) or pIRES2-EGFP (DLD-1-EGFP; control) plasmids were used for both BrdU incorporation and cell growth assays as described in Materials and methods. All results are expressed as the mean ± s.e.m. of eight samples. *$P<0.01$ *vs* control (DLD-1-EGFP cells) group.

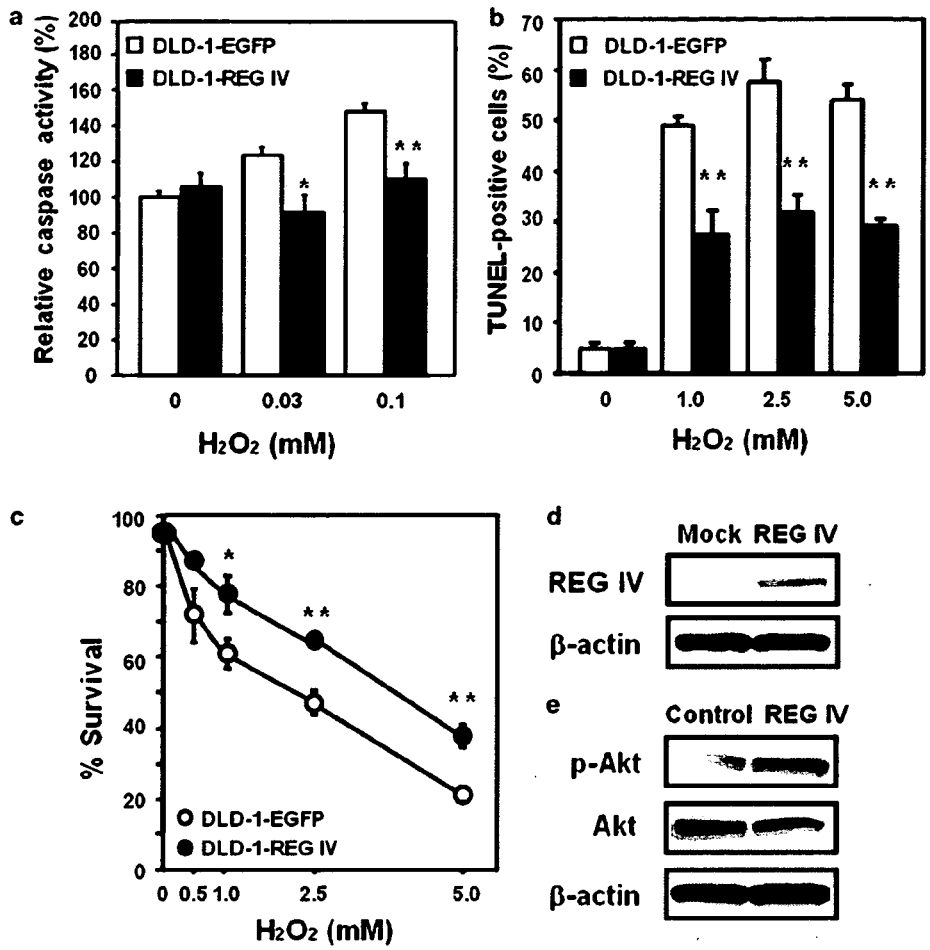

Figure 7 Effects of *REG IV* gene induction on H_2O_2-induced apoptosis and cell death of human colon cancer cells. DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) or pIRES2-EGFP (DLD-1-EGFP; control) plasmids were treated with different concentration of H2O2 for 2 h and assessed for caspase activity (**a**), TUNEL positivity (**b**), and cell survival (**c**) as described in Materials and methods. (**d**) Source of REG IV protein. Human embryonic kidney HEK293T cells were transfected with a human REG IV cDNA expression plasmid or a control plasmid, and the medium conditioned by these cells was collected. Release of REG IV protein (approximately 17 kDa) into the conditioned medium was confirmed by Western blot analysis with an anti-human REG IV polyclonal antibody. (**e**) Effect of REG IV conditioned medium on phosphorylation of Akt. All results are expressed as the mean ± s.e.m. of four samples. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control (DLD-1-EGFP cells) group at the same dose point.

by Kämäräinen[17] and Oue *et al*,[18] and suggests functional roles for REG IV protein in normal colonic mucosa. However, in UC tissues REG IV expression is abundantly enhanced, and the distributions of positivity for REG IV and chromogranin A were apparently distinct. Although we cannot explain this discrepancy, it is temping to speculate that a proportion of REG IV-positive cells may originate from non-endocrine cells in the UC mucosa. On the other hand, the distribution of REG IV-positive cells is relatively similar to that of Ki67-positive cells, suggesting that REG IV expression may be associated with proliferative behavior of epithelial cells in the UC mucosa. Interestingly, by using gene-chip analysis, several investigators have recently reported that a number of REG family proteins are markedly upregulated in the colonic mucosa of patients with UC.[19,20] Indeed, we have also shown enhancement of *REG Iα* gene expression in the colonic tissue of UC patients.[12] Moreover, others have reported an increase of *REG III* gene expression in UC mucosa.[21,22] Thus, our present study showing that the *REG IV* gene is also upregulated in the UC colonic mucosa strongly suggests generalized upregulation of *REG* family genes in the colonic mucosa of UC patients.

In addition to *REG IV* gene expression, we found in this study that the expression of both *bFGF* and *HGF* mRNA was significantly enhanced, and moreover that *TGF-α* mRNA expression tended to be increased in UC tissues. Thus, in addition to REG family proteins including REG IV, many growth factors appear to be involved in repair of the injured mucosa of patients with UC. Then, it may be interesting to ask why these various growth factors are concurrently upregulated in UC tissues. In this regard, we found in the present study that the level of *REG IV* expression showed significant

positive correlations with those of *bFGF* and *HGF*, although the *REG IV* gene is expressed in colonic epithelial cells while bFGF and HGF are produced mainly by interstitial mesenchymal cells.[23–26] These findings may indicate the presence of mutual interaction between growth factors in the epithelium and those in the interstitial cells.

In previous studies, we and others have demonstrated that the *REG Iα* gene is involved in inflammatory diseases[27,28] and that its expression is enhanced by proinflammatory cytokines such as IFN-γ and IL-6.[9,12,29–31] On the other hand, others have also demonstrated enhancement of *REG III* gene expression by several cytokines.[32,33] Accordingly, we also examined whether *REG IV* gene expression is stimulated by various proinflammatory cytokines. However, in contrast to *REG Iα* or *REG III* expression, we found that none of TNF-α, IL-6, IL-8, IFN-γ, or IL-1β had any stimulatory effect on *REG IV* gene expression in the human colon cancer cell line SW403. Therefore, we next examined the effects of various growth factors on *REG IV* expression because, as mentioned above, we had observed that the expression of not only *REG IV* but also that of various *growth factor* genes was upregulated, and that *REG IV* expression was positively correlated with *bFGF* and *HGF* expression in the colonic mucosa of UC patients. Interestingly, we found that stimulation with not only bFGF and HGF but also EGF and TGF-α, clearly enhanced the expression of *REG IV* mRNA in SW403 colon cancer cells. These growth factors are known to utilize the MAPK signaling cascade as their common signaling pathway. Indeed, we have shown in this study that the increase of *REG IV* gene expression by bFGF and HGF was dependent on activation of the MAPK pathway. Thus, since we found a positive correlation between expression of the *REG IV* gene and that of the *bFGF* and *HGF* genes in the UC colonic mucosa, it appears likely that the enhanced expression of the *REG IV* gene is at least partly due to upregulation of bFGF and HGF in the mesenchymal cells. On the other hand, since the expression of the EGF receptor or other growth factor receptors may be upregulated in UC tissues,[3,6] not only growth factors but also their receptors are likely responsible for *REG IV* expression. In this context, as described above, we previously reported that cytokines such as IFN-γ and IL-6 are responsible for the enhanced expression of REG Iα in the UC colonic mucosa.[12] Thus, it is tempting to hypothesize that expression of the *REG Iα* and *REG IV* genes is regulated specifically by different molecules, cytokines and growth factors. Interestingly, Bishnupuri *et al*[34] recently reported that REG IV protein activates the EGF receptor/Akt/AP-1 signaling pathway in human colon cancer cell lines. Thus, because EGF and TGF-α enhanced *REG IV* gene expression in this study, it is tempting to hypothesize that a positive functional loop exists between EGF/TGF-α and REG IV protein.

In this study, although we examined only four patients with colitic cancer, our data clearly revealed that *REG IV* is overexpressed not only in the dysplastic epithelial cells but also in colitic cancer cells of UC patients, suggesting the importance of REG IV in the development of colitic cancer from UC mucosa. In this regard, we demonstrated that *REG IV*-overexpressing colon cancer cells gain significant growth ability. Thus, similar to REG Iα protein, REG IV may also be involved in the development of colitic cancer through its growth-promoting action, although it still remains to be elucidated whether REG IV protein acts as a trophic factor on normal colonic epithelial cells. REG family proteins are known to have a similar structure to C-type lectins that possess various biological functions, including cell recognition, cell migration, cell growth and cell adhesion.[35,36] Therefore, in addition to its growth-promoting action, REG IV together with other REG family proteins may be involved in the development of colitic cancer by exerting a variety of actions. Indeed, similar to REG Iα, we have shown that REG IV protein has not only a mitogenic but also an antiapoptotic effect on colon cancer cells and exerts its effects at least in part by activation of Akt signaling. Thus REG family proteins may commonly act as mitogenic and/or antiapoptotic factors in the development of colitic cancer although their regulatory effects on gene expression may differ.

In summary, we have shown that the *REG IV* gene is expressed in not only epithelial cells of the UC mucosa but also dysplastic epithelial cells and colitic cancer cells. The expression of *REG IV* and other *growth factor genes* was concurrently enhanced, and *REG IV* expression was positively correlated with *bFGF* and *HGF* expression in UC mucosa. *In vitro* studies demonstrated that *REG IV* gene expression was enhanced by bFGF and HGF via the MAPK-dependent pathway. Moreover, *REG IV*-overexpressing colon cancer cells gained significant growth ability. Taken together, these results suggest that REG IV and other growth factors may act cooperatively to promote epithelial cell growth in the UC-colitic cancer sequence.

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Importance of Early Detection of Cytomegalovirus Infection in Refractory Inflammatory Bowel Disease

To the Editor:

Cases of inflammatory bowel disease (IBD) associated with cytomegalovirus (CMV) infection is a very important issue for gastroenterologist.[1,2] In patients with IBD refractory to conventional therapy, we should always consider the possibility of CMV infection. We note with great interest the article by Dimitroulia et al.[3] Data for patients with IBD who were investigated in that study demonstrated that the CMV genome in intestinal tissue was detected by polymerase chain reaction (PCR) in 32.9% of total IBD patients, while the CMV genome in the blood was detected in 27.1% of these patients. A positive ratio of CMV genome in intestinal tissue is higher in patients with ulcerative colitis (UC) than in those with Crohn's disease. Moreover, 5.9% of IBD patients had detectable CMV genome in their intestinal samples but not in their blood. On the other hand, in the control group who had no inflammatory disease, 11.9% of individuals had a detectable CMV genome in their blood, but 2.2% in their intestine.

The authors suggested that a potential role of CMV in the mucosal immune disturbance of IBD might be suspected because CMV infection occurred in intestinal tissue more dominantly than in the blood. However, they described that a definitive causal role of CMV in exacerbating IBD is still unknown. The detection of the CMV genome in colonic tissue may not necessarily indicate active CMV infection because infection usually results from reactivation of latent virus.

Recently, we clearly demonstrated that the detected copy number of CMV DNA by our PCR method is higher in the inflamed colonic tissue than that in noninflamed colonic tissue in patients with UC refractory to immunosuppressive therapy.[4] Moreover, our data demonstrated that all three patients with UC were positive for the CMV genome in inflamed colonic tissue alone, although neither CMV antigenemia nor CMV inclusion bodies were detected. In addition, we investigated more patients with refractory UC. As a result, 18 (94.7%) of 19 patients with refractory UC who were positive for CMV DNA in inflamed colonic mucosa by our PCR method had neither CMV inclusion body nor CMV antigenemia. All patients were successfully treated with antiviral therapy (unpubl. data). These results strongly support that CMV infection was involved in the deterioration of patients with UC and the investigation of CMV DNA in inflamed colonic tissue by the PCR method is necessary for early detection of CMV infection. Thus, both Dimitroulia's and our data included important points in considering diagnosis and therapy for patients with moderate or severe IBD who might have concomitant CMV infection.

In summary, we strongly believe that CMV infection is involved in exacerbation of patients with IBD. Therefore, early detection of CMV infection in intestinal tissue is absolutely necessary for developing a therapeutic strategy. Quantitative real-time PCR assay in gastrointestinal tissue is a promising modality for the diagnosis of CMV infection.

Hiroshi Nakase MD, PhD
Takuya Yoshino, MD
Satoru Ueno, MD
Norimitsu Uza, MD
Sakae Mikami, MD
Minoru Matsuura, MD, PhD
Tsutomu Chiba, MD, PhD
Department of Gastroenterology & Hepatology
Graduate School of Medicine
Kyoto University
Kyoto, Japan

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INTERNAL MEDICINE

□ CASE REPORT □

Rescue Therapy with Tacrolimus for a Patient with Severe Ulcerative Colitis Refractory to Combination Leukocytapheresis and High-Dose Corticosteroid Therapy

Hiroshi Nakase, Sakae Mikami, Minoru Matsuura, Satoru Ueno, Norimitsu Uza, Satoko Inoue, Hiroshi Kitamura, Katsuhiro Kasahara, Takuya Yoshino, Yasuhiro Takeda and Tsutomu Chiba

Abstract

A19-year-old man complaining of severe diarrhea and hematochezia was admitted to our hospital. Endoscopic findings and laboratory data revealed that he had ulcerative colitis (UC). Despite combination therapy with high-dose corticosteroids and intensive granulocytapheresis, his condition did not improve. Therefore, we initiated tacrolimus therapy. Intravenous administration of tacrolimus with a trough level of 10 to 15 ng/ml relieved his abdominal symptoms within 1 week. The patient experienced no UC relapse 1 year after treatment with oral tacrolimus. Tacrolimus is a promising therapy for patients with UC refractory to the combination of high-dose corticosteroids and leukocytapheresis.

Key words: ulcerative colitis, tacrolimus, leykocytapheresis

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Introduction

Tacrolimus is a macrolide antibiotic isolated from *Streptomyces tsukubanesis* that has immunomodulatory properties; it is efficacious and widely used for the prevention of allograft rejection in patients undergoing liver transplantation (1). Although its action is similar to that of cyclosporin (CyA), the immunosuppressive effect is 10 to 20 times greater in vivo than that of CyA and its intestinal absorption is more reliable, even in the presence of gastrointestinal disease (2). Therefore, much attention has been directed to tacrolimus for patients with inflammatory bowel disease (IBD) that are refractory to conventional therapy. In several uncontrolled studies, tacrolimus improved fistulizing Crohn's disease (CD) and steroid-refractory ulcerative colitis (UC) (3, 4). A recent randomized control study demonstrated the efficacy and safety of oral tacrolimus for inducing remission of refractory UC (5). Here, we report a patient with severe UC which was refractory to combination high-dose corticosteroids and leukocytapheresis therapy and was successfully treated with tacrolimus.

Case Report

A 19-old-year man with no medical history was admitted to our hospital because of high fever and frequent bloody diarrhea. Physical examination revealed localized tenderness at the left lower abdomen and increased bowel sounds. His temperature was 38℃. The following pathologic laboratory findings were noted: white blood cell count 11600/mm^3, C-reactive protein (CRP) 14 mg/dl, and serum albumin 2.9 g/dl (Table 1). Repeated blood cultures and testing for parasitic and bacterial bowel pathogens were negative. Colonoscopy revealed dull colonic mucosa and an erythematous pattern with a granular texture and gross pitting, and a blurred vascular pattern. Computed tomography showed increased wall thickness throughout the entire colon and increased vascularization within the bowel walls. The patient was diagnosed with severe total type UC. Under this diagnosis, daily therapy with 50 mg of prednisolone (PSL), along with 3000 mg mesalamine was started. Despite this

Department of Gastroenterology & Hepatology, Graduate School of Medicine, Kyoto University, Kyoto
Received for publication September 2, 2006; Accepted for publication January 25, 2007
Correspondence to Dr. Hiroshi Nakase, hiropy@kuhp.kyoto-u.ac.jp

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Table 1. Laboratory Data on Admission

Hematology			Normal range
WBC	11600/mm^3		3000-8500
Hb	11.8 g/dl		12.5-16.4
Plt	280000 /mm^3		167000-364000
Blood chemistry			
TP	6.7	g/dl	6.3-8.1
Alb	2.9	g/dl	3.9-5.1
GOT	17	IU/l	13-33
GPT	12	IU/l	8-42
ChE	157	IU/l	201-436
BUN	6	mg/dl	8-22
Cr	0.8	mg/dl	0.6-1.1
Na	137	mEq/L	136-144
K	3.5	mEq/L	3.6-4.8
CRP	14	mg/dl	0.2<

therapy, the patient's condition worsened. We then initiated treatment with 60 mg PSL and methylprednisolone pulse therapy (1000 mg for 3days). His condition improved 2 weeks after initiating this therapy. When the dose of PSL was tapered to 50 mg per day, however, his condition deteriorated again. He became increasingly anemic and hypoalbuminemic, and both blood in the stool and bowel movements increased. We thought that additional mesalamine enema might worsen the patient's symptoms and therefore did not perform it. Next, we started intensive granulocytapheresis (G-CAP) twice a week as additional therapy. Although we performed combination therapy with PSL and G-CAP six times, his condition did not improve. Colonoscopy revealed deep longitudinal ulcerations with mucosal erythema and edema at the sigmoid colon (Fig. 1). At this time, cytomegalovirus (CMV) antigenemia was negative. Histology and polymerase chain reaction method to detect CMV in a colonic biopsy specimen revealed no existence of concomitant CMV infection. We considered that it would be necessary to use immunosuppressive therapies with a strong and rapid onset of action for this patient because he was refractory to intensive G-CAP and high-dose corticosteroid therapy.

After informed consent was obtained from the patient and his family, tacrolimus was given by continuous intravenous infusion to adjust the serum trough levels of tacrolimus from 10 to 15 ng/ml. One week after initiating intravenous administration of tacrolimus, his abdominal pain disappeared and CRP became negative (Fig. 2). We then switched to oral administration of tacrolimus with the same trough level. Eight weeks after initiating the oral tacrolimus therapy, the administration of PSL was tapered off and the patient's condition was completely improved. Colonoscopy revealed regenerating epithelium at the sites of previous ulcerative lesions (Fig. 3). During this therapy, the patient did not experience any serious side effects other than tremor. We tapered the dose of tacrolimus to achieve a trough level of 5 to 10 ng/ml and he remained in remission 3 months later. One year later, the patient is still in remission without any side effects and the laboratory data show no signs of active inflammation.

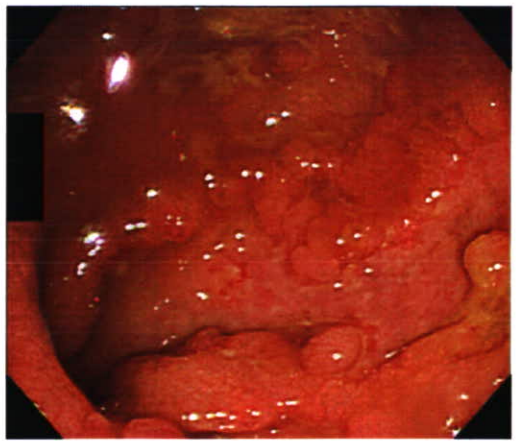

Figure 1. Colonoscopic view of the colonic ulcerations before administering tacrolimus.

Discussion

This is a case report of a 19-year-old Japanese patient with severe UC that was successfully treated by intravenous and oral administration of tacrolimus. The initial therapy for patients with moderate or severe UC is a combination of oral mesalamine and corticosteroids. Recently, the therapeutic effect of GCAP for active UC was reported in Japan. G-CAP is a new therapy in which granulocytes and monocytes are selectively absorbed by a G-1 Adacolumn. Naganuma et al reported that G-CAP therapy is a promising option for patients with moderate UC that are refractory to conventional therapy, with regard to reducing and avoiding PSL readministration, but that five sessions of G-CAP is not very effective for patients with severe UC (6). In this case, we intensively performed G-CAP twice per week to reduce the colonic inflammation, because the patient did not respond to high-dose PSL administration. This combination therapy, however, was not effective for this patient. In addition, endoscopic findings revealed deep longitudinal ulcer, which was suggestive of impaired mucosal healing. We therefore started intravenous administration of tacrolimus, for expecting rapid onset of action and letting this patient go into remission as soon as possible, because azathioprine may be ineffective in this active phase for its delayed onset of action.

Generally, intravenous administration of CyA is effective as a rescue therapy for patients with severe UC. The rapid

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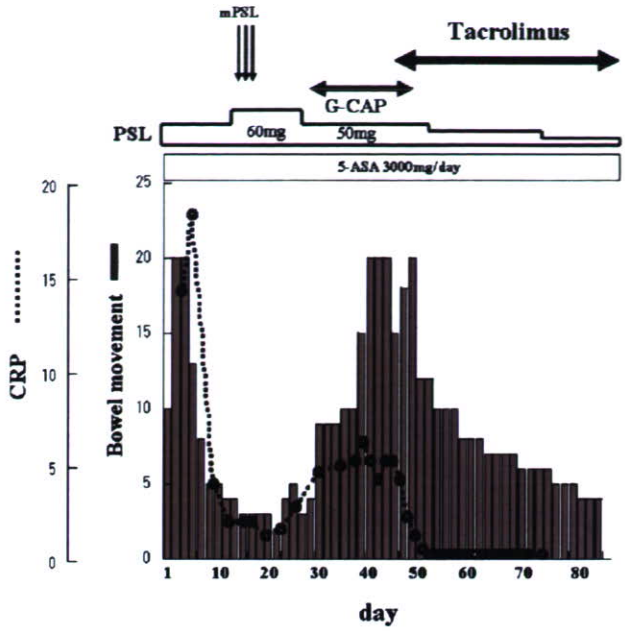

Figure 2. Clinical course of the patient successfully treated with tacrolimus.

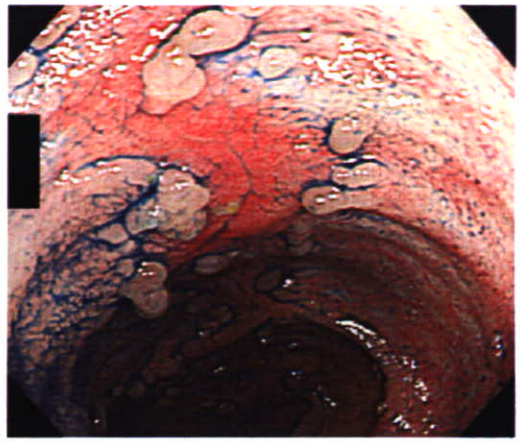

Figure 3. Colonoscopic view showing the appearance of regenerating epithelium at the previously ulcerated sites after tacrolimus administration.

efficacy of CyA to avoid emergency colectomy is approximately 50% to 80% in UC, but 35% to 67% of patients eventually undergo surgery (7-10). In addition, maintenance of remission with CyA requires high doses that are frequently associated with significant side effects such as gingival hyperplasia, hypertrichosis, hypertension, diabetes, nephrotoxicity, and neurotoxicity (11).

Another major disadvantage of CyA is the necessity to administer it intravenously to achieve sufficient, stable levels due to its variable intestinal absorption. The original oil-based oral formulation of CyA is characterized by high intra- and inter-patient's pharmacokinetic variability and poor bioavailability in patients with diarrhea, thereby preventing the stable blood levels of CyA (12).

Tacrolimus, however, is well-absorbed orally, compared to intravenous administration, even in severe colitis (4, 13). Moreover, recent evidence from transplant patients suggests that tacrolimus is superior to CyA with respect to immunosuppressive potency and has a lower incidence of side effects (14, 15). Based on these findings, we selected tacrolimus for this patient.

Tacrolimus has immunosuppressive properties similar to CyA, but is approximately 100 times more potent than CyA (16). Tacrolimus interacts with the calmodulin-dependent serine/threonine phosphatase calcineurin by binding of the immunophilin FKBP12. The main action is an abrogation of the translocation process of the nuclear factor of activated T-cells. This leads to a decrease in interleukin-2 levels, which in turn reduces the activation and proliferation of T cells (17). As for the therapeutic effects of tacrolimus in patients with IBD, some published case reports and uncontrolled studies suggest that tacrolimus therapy is beneficial in patients with steroid-resistant IBD or perianal fistulating CD (3, 4, 18, 19). A recent randomized study demonstrated dose-dependent efficacy and safety of oral tacrolimus for remission-induction therapy of refractory UC (5). Of refractory UC patients, that had been treated with a high trough concentration of tacrolimus (10～15 ng/ml), 68.4% had an improved disease activity index score with a reduction of more than four points within 2 weeks. Moreover, 20% of patients with refractory UC treated with such a high trough level of tacrolimus went into remission. These results suggest that the optimal treatment range, in terms of efficacy, for the induction of remission is 10 to 15 ng/ml. Therefore, we started tacrolimus therapy with aiming the trough level of 10～15 ng/ml, which resulted in the rapid improvement of the present patient's condition. In this case, although the patient has continued tacrolimus therapy for nearly 2 years, he has not experienced any serious side effects. He has been maintained in clinical remission with a trough level of tacrolimus of 5 to 10 ng/ml. There are currently only a few reports on the long-term efficacy and safety of tacrolimus as a maintenance treatment for patients with UC (18). In this regard, further clinical trials are needed to evaluate whether tacrolimus is an optimal drug for maintenance therapy for patients with UC (19, 20).

In conclusion, we report a patient with UC refractory to combination of corticosteroid and intensive G-CAP therapy, that was successfully treated with tacrolimus. Controlled trials comparing tacrolimus with CyA in patients with UC are not yet available, and it is therefore difficult at present to conclude which drug is superior. Tacrolimus, however, is an alternative option for patients with UC that are refractory to conventional therapy.

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GASTROENTEROLOGY

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

Satoko Inoue, Hiroshi Nakase, Minoru Matsuura, Satoru Ueno, Norimitsu Uza, Hiroshi Kitamura, Sakae Mikami, Hiroyuki Tamaki, Katsuhiro Kasahara and Tsutomu Chiba

Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

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

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Correspondence
Dr Hiroshi Nakase, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University Hospital, Kawara-cho 54, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Email: hiropy@kuhp.kyoto-u.ac.jp

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

Journal of Gastroenterology and Hepatology 22 (2007) 984–988

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

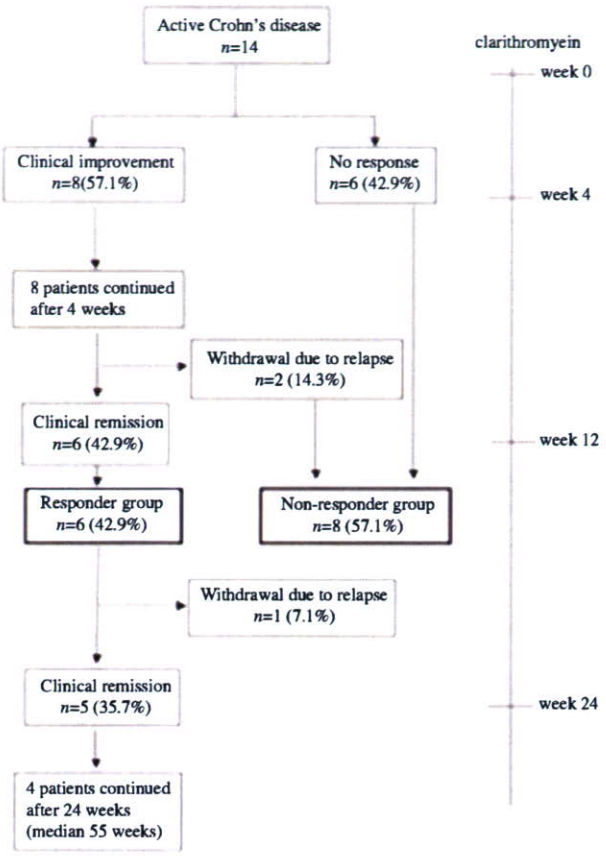

Figure 1 Clinical response in patients with active Crohn's disease receiving clarithromycin ($n = 14$). Clinical activity was evaluated by the Crohn's Disease Activity Index (CDAI) at entry, and at 4, 12, and 24 weeks after starting clarithromycin.

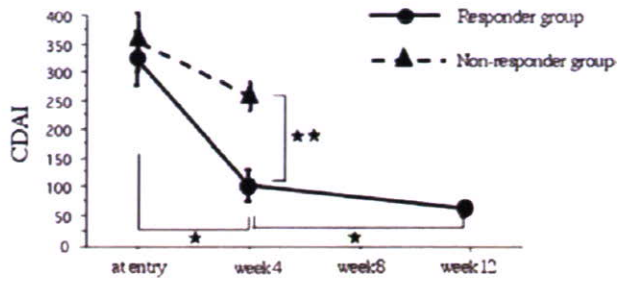

Figure 2 Change in the Crohn's Disease Activity Index (CDAI) score at entry, and at 4 and 12 weeks after starting clarithromycin in responder ($n = 6$) and non-responder ($n = 8$) groups. $^{*}P < 0.05$; $^{**}P < 0.01$.

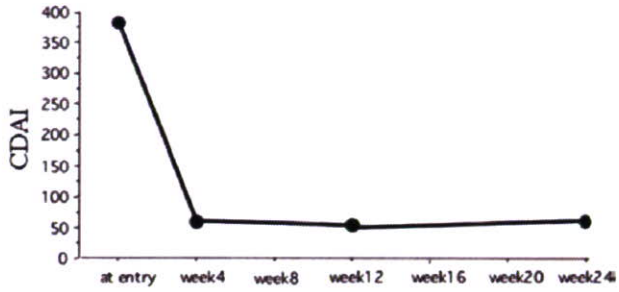

Figure 3 Change in the Crohn's Disease Activity Index (CDAI) score at entry, and at 4, 12, and 24 weeks after starting clarithromycin in one responder. In spite of treatment with azathioprine, his CDAI score was high at entry. After he started clarithromycin therapy, he achieved remission within 4 weeks,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