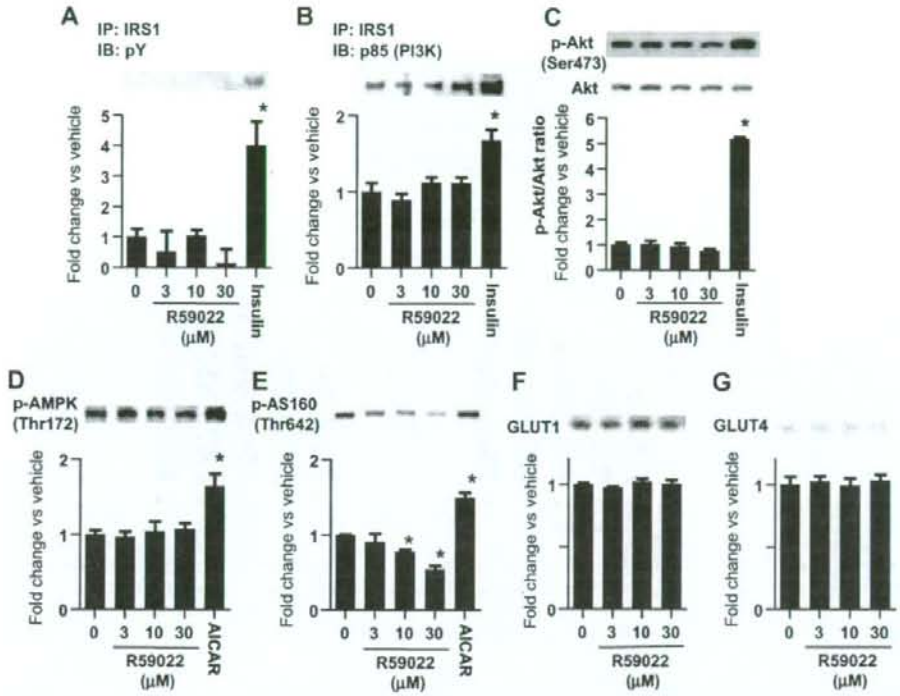

Fig. 2. Effect of R59022 on (A) IRS1 tyrosine-phosphorylation, (B) IRS1/PI3K association, (C) Akt phosphorylation, (D) AMPK phosphorylation, (E) AS160 phosphorylation, (F) GLUT1 protein and (G) GLUT 4 protein expression. C2C12 myotubes were treated with several doses of R59022 for 24 h. For positive control, insulin (100 nM for 15 min) or AICAR (100 μM for 30 min) was used. The protein was extracted for later analysis. Data are calculated by fold change versus vehicle and expressed as means ± SE ($n = 3$). $^*P < 0.05$, when compared vehicle only. Representative blot was shown on the upper side of the graph.

relevant in R59022-induced stimulation of glucose transport.

We next tried to investigate the mechanism of R59022-induced p38 activation in the skeletal muscle cells. Because two mitogen-activated protein kinase kinases, MKK3, and MKK6 are immediate upstream activators for p38 [18], the effect of R59022 on MKK3/6 phosphorylation in C2C12 myotubes was examined. R59022 as well as insulin significantly stimulated phosphorylation of MKK3/6 (Fig. 4B). The stimulation of MKK3/6 phosphorylation was dose-dependently observed by R59022.

Discussion

In the present study, we examined whether DGK may be implicated in glucose transport in skeletal muscle cells because the skeletal muscle expresses DGK [3]. Since several research groups used C2C12 cells to analyze glucose transport in the skeletal muscle cells [19,20], the C2C12 cells were selected in this study. We first examined whether C2C12 myotubes express several DGK isoforms mRNAs and showed that C2C12 myotubes expressed DGKα, δ, ε, ζ, and θ isoforms, indicating that C2C12 myotubes express all five subtypes (type 1–5) of DGK. Earlier investigators demonstrated that C2C12 mouse myoblasts expresses DGKδ and ζ [21,22]. The present study therefore provided an evidence that C2C12 myotubes express not only DGKδ and ζ, but DGKα, ε, and θ isoforms. In the following experiments, we used C2C12 myotubes as a model to investigate a role of DGK.

Next, we examined the effect of DGK inhibition by a specific inhibitor on glucose transport in the C2C12 myotubes to clarify a role of DGK in glucose transport in the skeletal muscle cells. As clearly demonstrated in this study, R59022 significantly stimulated glucose transport in skeletal muscle cells in dose- and time-dependent manners, suggesting that DGK inhibition would increase glucose transport in skeletal muscle cells. In other words, glucose transport would be suppressed by DGK in skeletal muscle cells. DGK phosphorylate the second-messenger DG to PA [3–6]. DGKs play a role in lipid–protein and protein–protein interactions in various signaling pathways dependent on DG and/or PA production. DGK is therefore believed to be activated at the plasma membrane where DG is generated. Strålfors et al. have demonstrated that DG enhanced glucose transport in adipocytes [23]. It has been

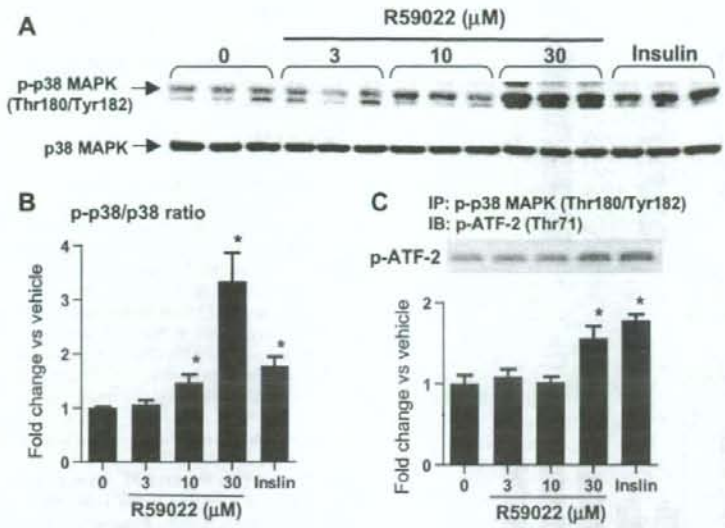

Fig. 3. Effect of R59022 on p38 activity in C2C12 myotubes. C2C12 myotubes were treated with several doses of R59022 for 24 h. Insulin was used at 100 nM for 15 min for positive control. (A) Representative immunoblot with phospho-p38 (Thr180/Tyr182) antibody (upper panel) or total p38 antibody (lower panel) were shown. (B) Results from A were quantified by densitometry and data represent means ± SE (n = 3). (C) *In vitro* kinase assay of p38. Total cell lysates were immunoprecipitated with phopsho-specific p38 antibody and then the products were performed kinase assay with recombinant ATF-2, a substrate of p38. Activity of p38 was determined by blotting the assay products with phospho-ATF-2 (Thr71) antibody. Representative blot was shown on the upper side of the graph. Data are calculated by fold change versus vehicle and expressed as means ± SE ($n = 3$). $^*P < 0.05$, when compared vehicle only.

furthermore shown that phorbol ester, a DG analogue [24], stimulates muscle glucose transport [25]. These results suggest that DG stimulates glucose transport. Because R59022 is an inhibitor of DGK, DG should be accumulated by treatment with R59022. Although the present study did not confirm that R59022 indeed increased DG level in the cells, these evidences led us to speculate that DG accumulation might mediate at least in part the R59022-induced stimulation of glucose transport in C2C12 myotubes. In any event, these evidences suggested that DGK might be involved in glucose transport. As we have clearly demonstrated in this study, C2C12 myotubes used in this study express at least five isoforms of DGK. Among these isoforms of DGK, further study should be needed to clarify which isoform(s) is involved in the regulation of glucose transport.

Next, we tried to clarify the mechanism by which R59022 stimulates glucose transport in C2C12 myotubes. Since the mechanism of glucose transport stimulated by insulin is well characterized [14], we tested a possibility whether R59022 may stimulate glucose transport through a mechanism similar to insulin signaling in skeletal muscle cells. An IRS1–PI3K–Akt pathway is well established as a signaling that mediates insulin-induced stimulation of glucose transport in skeletal muscle cells [14]. It has been demonstrated that muscle contraction is a potent stimulator for glucose transport independently of the IRS1–PI3K–Akt pathway by activating AMPK [15]. We therefore examined the above possibility that an IRS1–PI3K–Akt pathway or muscle contraction signal, or both might mediate the R59022-induced stimulation of glucose transport in C2C12 myotubes. The present study however failed to support the above possibility.

Marshall et al. have demonstrated that glucose transport is stimulated in GLUT 1 or 4 transgenic mice [26], indicating that increased expression of glucose transporter, GLUT, stimulates glucose transport. We therefore examined the effect of R59022 on expression of GLUT1 and 4. As demonstrated in this study, R59022 failed to increase GLUT1 and 4 expression in C2C12 myotubes, suggesting that the stimulated glucose transport by R59022 is not associated with the expression of GLUT in the skeletal muscle.

p38 is involved in glucose transport in the skeletal muscle cells [17]. For instance, activation of p38 by anisomycin, a specific activator for p38, stimulates p38 phosphorylation and increases glucose transport [27]. In contrast, inhibition of p38 by SB203580 suppresses glucose transport in skeletal muscle cells [28]. Thus, p38 plays a vital role in glucose transport in the skeletal muscle cells. The present study demonstrated that R59022 significantly stimulated p38 phosphorylation, its kinase activity and glucose transport and SB203580 blocked the R59022-induced stimulation of glucose transport in C2C12 myotubes, strongly suggesting that p38 activation may mediate the R59022-induced stimulation of glucose transport.

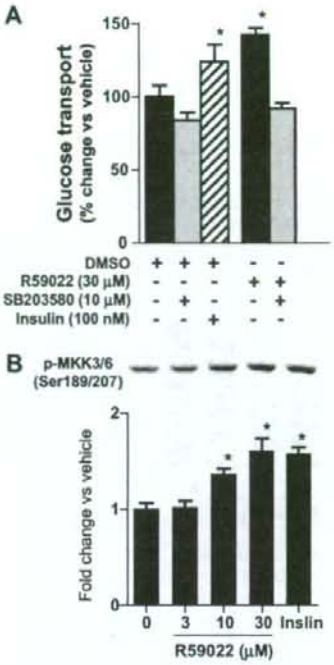

Fig. 4. (A) Effect of SB203580, a p38 inhibitor, on the R59022-induced stimulation of glucose transport in C2C12 myotubes. Glucose transport was assayed using [^{3}H]2-deoxyglucose for last 4 min. Data are expressed as means ± SE ($n = 4$). $^*P < 0.05$, when compared vehicle only. (B) Effect of R59022 on MKK3/6 phosphorylation. C2C12 myotubes were treated with several doses of R59022 for 24 h. Insulin was used at 100 nM for 15 min for positive control. Phosphorylation of MKK3/6 was analyzed by immunoblot using phospho-MKK3/6 (Ser189/207) antibody. Representative blot was shown on the graph. The bands were quantified and data are calculated by fold change versus vehicle and expressed as means ± SE ($n = 3$). $^*P < 0.05$, when compared vehicle only.

Because two mitogen-activated protein kinase kinases, MKK3, and MKK6 are immediate upstream activators for p38 [18], we made a hypothesis that MKK3/6 phosphorylation may be a upstream signaling mechanism to activate p38 by R59022. As demonstrated in the present study, R59022 increased MKK3/6 phosphorylation in C2C12 myotubes, suggesting an involvement of MKK3/6 in p38 activation by R59022. The R59022-induced stimulation of glucose transport, activation of p38 and phosphorylation of MKK3/6 were all observed by R59022 in the same dose range (10–30 μM), furthermore supporting the speculation that the MKK3/6-p38 signaling pathway mediates the R59022-induced stimulation of glucose transport in C2C12 myotubes.

In summary, the present study suggested that DGK may play a role in glucose transport in the skeletal muscle cells through modulating a MKK3/6-p38 signaling pathway.

Acknowledgment

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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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KEYWORDS: cell growth; colitic cancer; growth factor; REG; ulcerative colitis

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).

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) 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

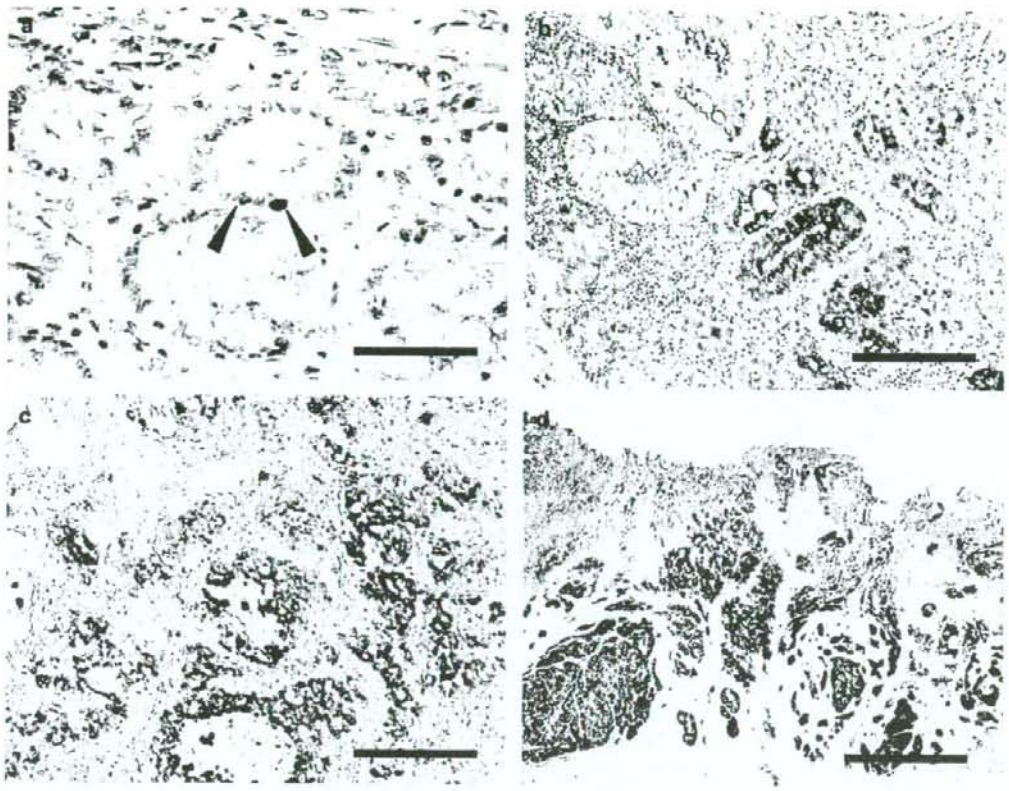

Figure 1 *In situ* hybridization of *REG IV* mRNAs in normal colon (**a**), UC (**b**), dysplasia (**c**), and colitic cancer (**d**) tissues. (**a**) Normal human colonic mucosa. Hybridized signals for *REG IV* mRNA (arrowheads) are evident in only a few epithelial cells in the crypts (bar = 50 μm).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

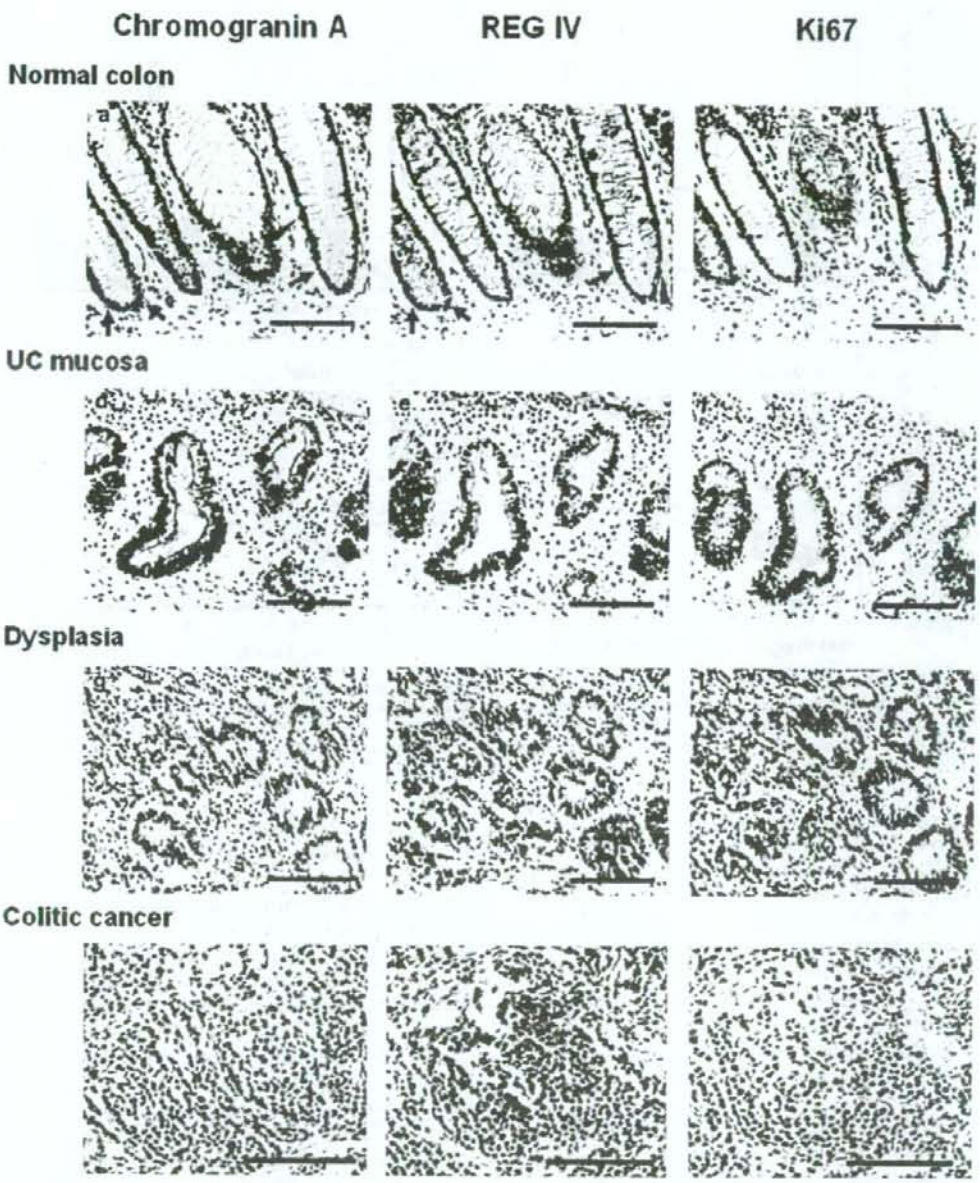

Figure 2 Serial immunostainings of REG IV protein, chromogranin A and Ki67 in normal colonic (**a–c**) and UC mucosa (**d–f**), and in dysplasia (**g–i**) and colitic cancer (**j–l**). In normal colonic mucosa, REG IV and chromogranin A were co-expressed in a few epithelial cells in the basal portion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

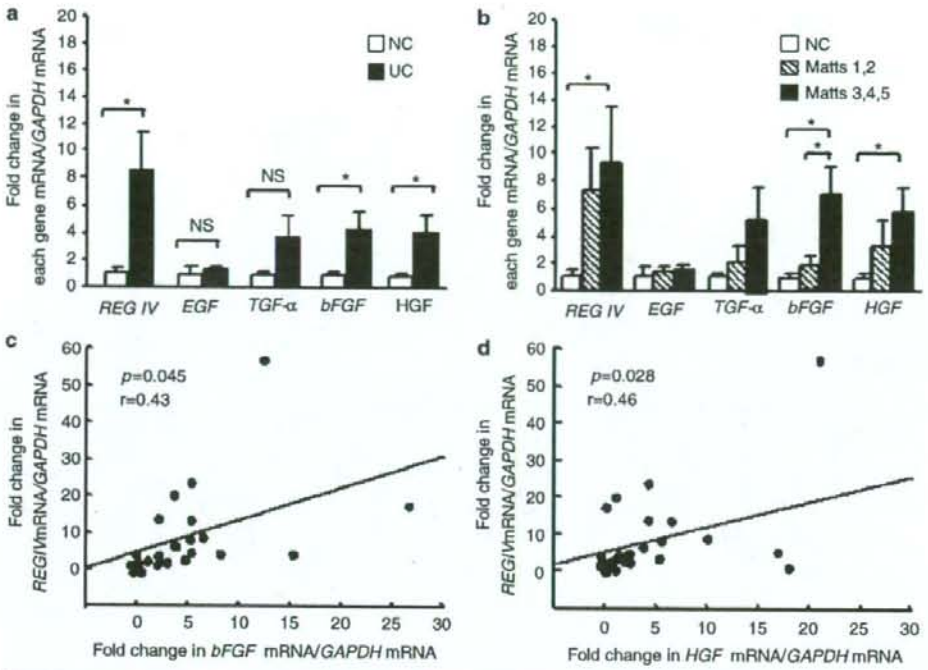

Figure 3 *REG IV* mRNA expression in UC tissues. (**a**) Expression levels of mRNA for *REG IV* and other *growth factors* in normal colon and UC tissues. (**b**) Comparison of mRNA expression levels for *REG IV* and other *growth factors* among UC groups subdivided by histological findings. Correlation 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).

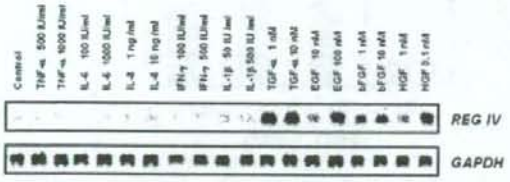

Figure 4 Effects of various cytokines and growth factors on *REG IV* mRNA expression in SW403 cells. Total RNA (20 μg) was extracted 12 h after stimulation with cytokines or growth factors and analyzed by Northern blotting using ^{32}P-labeled cDNAs for *REG IV* and *GAPDH* mRNA.

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

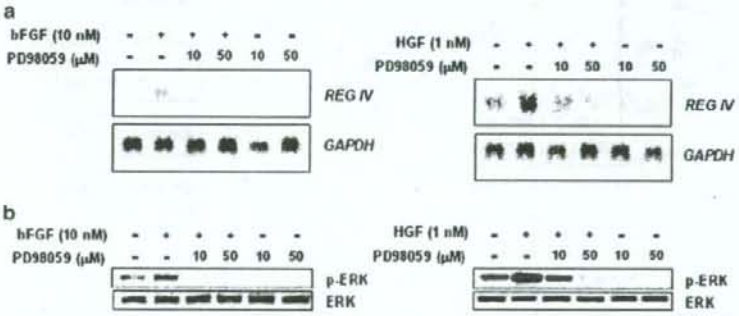

Figure 5 Effects of bFGF and HGF on *REG IV* gene expression via the MAPK pathway in SW403 cells. (**a**) The cells were preincubated with 10 or 50 μM PD98059 for 1 h and stimulated with 10 nM bFGF or 1 nM HGF for 12 h. Total RNA (20 μg) was extracted and analyzed by Northern blotting using ^{32}P-labeled 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.

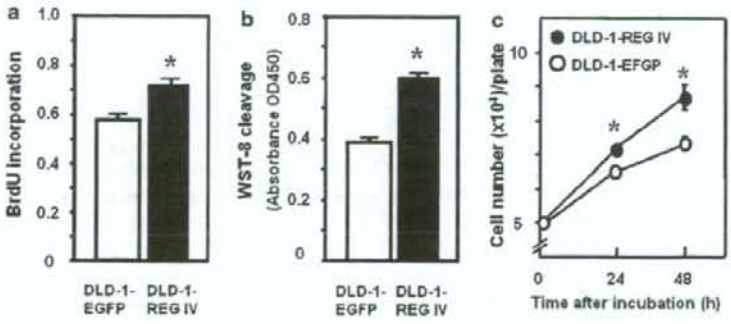

Figure 6 Effects of *REG IV* gene induction on BrdU incorporation by (**a**) and growth of (**b**, **c**) human colon cancer cells. DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) or pIRES2-EGFP (DLD-1-EGFP; control) plasmids were used for both BrdU incorporation and cell growth assays as 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.

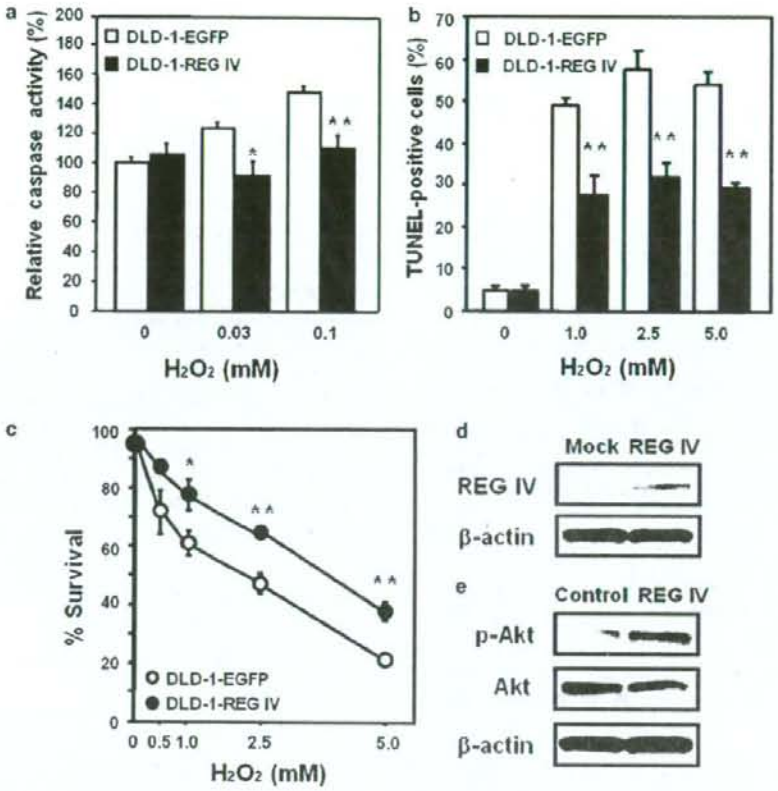

Figure 7 Effects of *REG IV* gene induction on H_2O_2-induced apoptosis and cell death of human colon cancer cells. DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) or pIRES2-EGFP (DLD-1-EGFP; control) plasmids were treated with different concentration of H2O2 for 2 h and assessed for caspase 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.

ACKNOWLEDGEMENT

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Clinical Significance of Serum p53 Antibodies in Patients with Ulcerative Colitis and Its Carcinogenesis

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Background: For early detection of ulcerative colitis (UC)-associated colorectal cancer (CRC), surveillance colonoscopy is recommended in UC patients at high risk. However, poor acceptability deteriorates its effectiveness and a suitable marker for selecting patients at high risk is needed. Here we evaluated clinical usefulness of the measurement of anti-p53 antibodies (Abs) by enzyme-linked immunosorbent assay (ELISA) using sera samples from UC patients.

Methods: Sera from 286 patients with UC, 82 patients with sporadic CRC, and 63 healthy controls (HC) were obtained. Serum anti-p53 antibodies were detected with ELISA. Immunohistochemical detection was also performed in patients who developed dysplasia or CRC.

Results: Serum p53 Ab was positive in 15.0% of UC, while it was positive only in 1.6% of HCs. In sporadic CRCs, 52.4% of 82 patients were positive. In UC patients with disease duration equal to or longer than 8 years, positivity of serum p53 Ab was significantly higher than those in patients with shorter duration. Eight of 13 (61.5%) UC patients with CRC or dysplasia were positive for serum p53 Abs, which was significantly higher than that in patients without neoplasia. All UC patients with CRC were positive for p53 staining, while 2 were negative for serum p53 Ab. Finally, levels of serum p53 Ab had fallen in 4 patients with CRC we could monitor after surgery.

Conclusions: This study revealed that p53 Ab developed in the progression of UC-associated CRC but not in all patients with neoplasia, suggesting that serological detection of p53 Abs by ELISA is not suitable in primarily selecting patients at high risk; however, it is helpful in salvaging patients who drop from a surveillance program.

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Key Words: ulcerative colitis, colorectal cancer, p53, enzyme-linked immunosorbent assay, surveillance

Ulcerative colitis (UC) is an inflammatory bowel disease of unknown etiology characterized by periods of remission and acute episodes of relapse that accompany severe inflammation in colonic mucosa.[1] The risk for colorectal cancer (CRC) increases in patients with chronic UC after 10 years and the cumulative CRC rate is 2% after 10 years, 8% after 20 years, and 18% after 30 years of the disease,[2] while it was reported to be relatively low in Japan, 0.5%, 4.1%, and 6.1%, respectively.[3] Risk factors for CRC in patients with UC include disease duration, early onset, extensive disease, complication of primary sclerosing cholangitis, severity of inflammation, and family history of sporadic CRC.[4,5] Since the development of CRC accounts for poor prognosis in patients with UC, a surveillance program using colonoscopy with random biopsies in patients with long-standing UC is currently recommended.[6,7]

Cancer surveillance is based on the hypothesis that repeated testing of a high-risk population will identify patients who have cancer or who have a potential risk for the development of cancer. Dysplasia in UC mucosa is not recognized only as a precursor of cancer, but an indicator for the simultaneous presence of cancer.[8–10] Dysplasia is usually patchy and develops from flat mucosa, and therefore it may be missed even with multiple biopsies. Random colonic mucosal biopsies have been recommended in surveillance colonoscopy; however, the number of biopsy specimens required is large and the cost of this procedure is very high.[11,12] Although the number of patients subject to surveillance is increasing year by year, all of such patients do not enroll in a surveillance program. Poor acceptability for surveillance colonoscopy and low patient compliance deteriorate the effectiveness of surveillance programs.[13] Moreover, patients who developed CRC out of a surveillance program had poor prognoses.[3] Therefore, a suitable marker for selecting patients

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TABLE 1. Characteristics of Study Populations

	HC (n = 63)	UC (n = 286)	CRC (n = 82)
Sex (M:F)	23:40	136:150	56:26
Age (mean ± SD, range)	52.9 ± 17.8 (25–88)	40.3 ± 14.6 (14–85)	65.5 ± 11.2 (31–91)

HC, healthy control; UC, ulcerative colitis; CRC, sporadic colorectal cancer; M, male; F, female; SD, standard deviation.

at high risk of developing CRC in UC, who would be strongly recommended for surveillance colonoscopy, is needed, even though it must be ideal that all UC patients with longer duration and extensive disease could take surveillance colonoscopy every year.

Molecular mechanisms underlying UC-associated neoplasia have been studied for years, but understanding of these mechanisms remains incomplete. It is likely that an accumulation of multiple genetic alterations leads to dysplasia and cancer in UC in a manner similar to that of sporadic CRC.[14] However, biological features differ between UC-associated CRC and sporadic CRC. Mutations in the *APC* gene and *ras* proto-oncogenes are not consistently present in UC-associated CRC in contrast to sporadic CRC resulting from an adenoma-carcinoma sequence pathway.[15–19] Conversely, mutation of the *p53* tumor suppressor gene is a frequent occurrence in early dysplastic UC lesions and precedes *p53* loss of heterogeneity.[20,21] Mutations of the *p53* gene are associated with, and likely precede, dysplasia and cancer,[22] whereas mutations within the *p53* gene in sporadic CRC are thought to occur at the adenoma-carcinoma transition.[23] Inactivation of the *p53* gene is usually caused by missense point mutations, most of which modify conformation and stability of p53 protein and lead to its accumulation in the nuclei of tumor cells. Although the most accurate procedure for analysis of p53 status is DNA sequencing, immunohistochemical analysis has been widely employed to screening p53 alterations in clinical and research fields. *p53* gene mutations with detection of the mutated protein products using immunohistochemistry offer promising possibilities as a test complementary to dysplasia for use in cancer surveillance programs.[24] Meanwhile, it has been shown that patients with various types of neoplasia have p53 antibodies (Abs) in their sera.[25,26] Recently, an approach for diagnosis of p53 alterations has been developed, which consists of detection of p53 Abs in sera from patients affected by a wide variety of cancers by enzyme-linked immunosorbent assay (ELISA).[27–29] Serological detection of p53 Abs is easier to perform and to repeat, does not require tumor material, and is of potential interest for monitoring patients with cancer.

We previously reported that interferon-inducible gene family 1-8U expression was significantly higher in UC-associated CRC tissues than in mucosa with mild inflammation in the same UC patients[30] and that UC-associated cancer or dysplasia had a higher percentage of microsatellite instability than nonneoplastic UC mucosa.[31] Although these findings could be good markers for distinguishing UC-associated CRC from nonneoplastic tissue, we consider that these are not suitable markers for selecting patients at high risk for the development of CRC in the clinical setting. In the present study we attempt to evaluate the clinical usefulness of measurement of anti-p53 Abs by ELISA using sera samples from UC patients with or without neoplasia.

PATIENTS AND METHODS

Study Population

This study was carried out according to the ethical standards of the institution's human investigation committee and the Helsinki Declaration of 2004 and was approved by the ethical committee at Keio University Hospital. All patients and healthy controls (HCs) were enrolled at Keio University Hospital between 2002 and 2005 and gave informed consent.

Blood samples were obtained by venipuncture from 286 patients with UC and 82 patients with sporadic CRC as positive disease control. UC patients with CRC who had undergone surgery were excluded. The diagnosis of UC was according to accepted criteria.[32,33] At the time of diagnosis, we excluded infectious colitis, radiation colitis, ischemic colitis, Crohn's disease, and intestinal Behçet's disease. All the patients with sporadic CRC were at advanced stage and treated surgically. Blood samples were also obtained from 63 HCs who were defined as subjects never diagnosed to have any cancer or UC by yearly medical examinations including fecal occult blood test, even though total colonoscopy was not performed in every HC subject.

Out of 286 UC patients, 136 (47.6%) were male and 150 (52.4%) were female, with a mean age of 40.3 years (range, 14–85); in patients with sporadic CRC, 56 (68.3%) were male and 26 (31.7%) were female, with a mean age of 65.5 years (range, 31–91); and in HCs, 22 (34.9%) were male and 41 (65.1%) were female, with a mean age of 49.9 years (range, 25–83) (Table 1).

In UC patients, smoking habits and family history of CRC were reviewed. Clinicopathological features of UC such as duration of the disease, extension of the disease, clinical disease activity (Truelove-Witts criteria[34]) at the time of

sample collection, extraintestinal complications, and medications were collected from the medical records. No patients were known to have previously had any other malignant or autoimmune disease.

All patients with UC had undergone routine colonoscopy or barium enema study within 1 year before blood sampling. We performed surveillance colonoscopy with random biopsies in addition to targeted biopsies for UC patients with long-standing and extensive colitis; however, only 7.9% of patients received surveillance colonoscopy every year in our institute. The study population included patients with UC not subjected to a surveillance program, and therefore a considerable ratio of patients with UC did not have so-called surveillance colonoscopy.

Among 286 UC patients, endoscopic and histological examinations revealed that 8 patients had CRC, 1 had high-grade dysplasia (HGD), and 4 had low-grade dysplasias (LGD) at the colorectum. The degree of dysplasia was diagnosed according to previously published criteria.[10] UC-associated CRC was staged according to the fifth edition of the American Joint Committee on Cancer staging system.[35] No other patients with UC were revealed to have any neoplasms at the colorectum and following up these patients after blood sampling revealed that none of these developed CRC or dysplasia. Those with UC-associated CRC who underwent surgery were subjected to repeated analyses of serum anti-p53 Abs after surgery.

Serum Samples

Whole blood was centrifuged at 1200*g* for 10 minutes and serum was stored in aliquots at −80°C until assayed.

ELISA for Anti-p53 Abs

ELISA for detection of anti-p53 Abs in serum was carried out with a commercially available ELISA kit (DIANOVA, Oncogene Research Products, Cambridge, MA). This ELISA utilizes microtiter plates precoated with recombinant human wildtype p53 protein. All samples were assayed in duplicate. Briefly, 100 μL per well of calibrators, negative control, or 1:100 diluted samples were added to a microtiter plate and incubated for 1 hour at room temperature (RT). Following incubation the plate was washed to rid it of any unbound material and 100 μL of a peroxidase-conjugated goat antihuman antibody, which bound any captured human p53 antibody, was added to the microplate and incubated for 1 hour at RT. After washing, 100 μL of chromogenic substrate tetramethylbenzidine was added to the wells. Enzymatic reaction was stopped after 30 minutes with 50 μL of 2 M HCl stop solution. Color reaction was measured immediately by absorption at 450 nm using a spectrophotometer. A calibration curve was constructed from specific signals of standards and from the levels of Abs indicated on standard vials.

For determination of the cutoff line we evaluated serum anti-p53 Abs of 63 HCs and defined 5.854 U/100 μL, mean value plus 2 standard deviation (SD), as the cutoff line for the Japanese population. Therefore, we judged positive for serum anti-p53 Ab in the present study when the anti-p53 Ab level was higher than 5.854 U/100 μL.

Immunohistochemistry

For those who were revealed to have dysplasia or CRC with colonoscopy, we also evaluated p53 status with immunohistochemistry using biopsied samples. Briefly, paraffin-embedded tissue samples were cut into serial sections 6 μm thick, placed on coated slides, and deparaffinized through a series of xylene and ethanol. Slides were then incubated with anti-p53 primary antibodies (DO-7; DAKO, Glostrup, Denmark), followed by biotin-conjugated goat antibody against mouse as secondary antibody (E0433; DAKO). The following steps were done using a standard ABC method (Elite ABC kit, Vectastain, Vector Laboratories, Burlingame, CA). 3,3-Diaminobenzidine was used as substrate for the peroxidase enzyme reaction (Vectastain). All slides were counterstained with hematoxylin and observed under a microscope (CH40; Olympus, Japan).

Statistical Analysis

Data are expressed as mean ± SD. A Mann-Whitney *U*-test was used for comparison of 2 groups. A chi-square test was used to evaluate the relationship between categorical variables. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of Patients with UC

Clinical characteristics of UC patients are summarized in Table 2. Routine colonoscopy or barium enema study was negative for dysplasia and CRC except in 13 patients who were shown to have dysplasia or CRC. None of the rest of the patients with UC have developed CRC or dysplasia since then.

Positivity of Serum p53 Abs

Among 63 HCs, titer of serum p53 Abs ranged from 0.00 U/100 μL to 6.76 U/100 μL and only 1 subject (1.6%) was considered positive for serum p53 Abs. In sporadic CRCs, titer of serum p53 Abs ranged from 0.00 U/100 μL to 444.19 U/100 μL and 43 of 82 patients (52.4%) were positive for serum p53 Abs, which was significantly higher than those in the HC group ($P < 0.0001$). Among 286 UC patients, serum p53 Ab levels ranged from 0.00 U/100 μL to 44.40 U/100 μL and 43 patients (15.0%) were positive for serum p53 Abs (Table 3). Although there was no significant difference in serum p53 Ab titers between the UC and HC groups, positivity of serum p53 Abs in the UC group was significantly

TABLE 2. Association Between Patient Characteristics and Serum p53 Ab Positivity

Characteristics	Patients No.	p53 Abs-positive No.	(%)	*P* Value
Sex				
Male	136	21	15.4	0.870
Female	150	22	14.7	
Smoking habits				
Nonsmokers	216	34	15.7	0.066
Smokers	25	0	0	
Ex-smokers	28	6	21.4	
(Unknown	17	3	17.6)	
CRC family history				
Positive	15	2	13.3	>0.99
Negative	271	41	15.1	
Age				
<37	138	22	15.9	0.370
≥37	148	21	14.2	

CRC, sporadic colorectal cancer.

higher than that in the HC group ($P = 0.0014$). Even if UC patients complicated with CRC were excluded, positivity of serum p53 Abs in the UC group (13.3%) was significantly higher than that in the HC group ($P = 0.0063$).

Serum p53 Abs and Clinicopathological Features in UC Patients

Characteristics of UC patients and numbers and percentages of p53 Abs-positive subjects are described in Table 4. No significant differences were found between p53-positive and sex, age, extent of disease, or clinical disease activity at blood sampling. However, 30 (20.8%) of 144 patients with disease duration equal to or longer than 8 years were positive for serum p53 Abs, while only 13 (9.2%) of 142 patients with durations less than 8 years were positive ($P = 0.0076$). If UC patients complicated with CRC were excluded, the difference between the 2 groups was not significant ($P = 0.053$). As for extent of disease, 8 (11.9%) of 67 patients with proctitis or proctosigmoiditis, 13 (15.5%) of 84 patients with left-sided colitis, and 21 (15.8%) of 133 patients with pan-colitis were positive for serum p53 Abs ($P = 0.7439$, not significant). In patients with disease duration equal to or longer than 8 years, there was a weak correlation between p53-positive and extent of disease (2 of 25 [8.0%] for proctitis or proctosigmoiditis, 9 of 50 [18.0%] for left-sided colitis, 18 of 68 [26.5%] for pan-colitis [$P = 0.1435$]).

In 286 UC patients we evaluated, endoscopic and histological examinations revealed 8 CRC, 1 HGD, and 4 LGD. Among these patients with neoplasms, 6 (75.0%) out of 8 patients with CRC and 2 (40.0%) out of 5 patients with dysplasia were positive for serum p53 Abs. Positivity of serum p53 Abs in UC patients who developed CRC was significantly higher compared with UC patients without CRC ($P = 0.0002$). The p53 Ab level of UC patients with CRC ranged from 1.5 U/100 μL to 44.4 U/100 μL, which was significantly higher than in patients without CRC ($P < 0.0001$; Fig. 1). The positivity of serum p53 Abs in UC patients with CRC (75.0%) was higher than that in sporadic CRC patients (52.4%), but it was not significant ($P = 0.2828$). The positive predictive value of positive p53 Ab for CRC in UC patients we evaluated was calculated at 14.0% (6/43).

Serum p53 Abs and p53 Overexpression by Immunohistochemistry

In all patients who had CRC or dysplasia, we evaluated p53 overexpression in tumor cells by immunohistochemistry using biopsied or surgical specimens. The results of immunohistochemical analysis of p53 are shown in Table 5. All UC patients with CRC were positive for p53 staining, while 2 were negative for serum p53 Ab. Of these 2 patients, 1 had far advanced CRC metastasizing to the liver.

We also evaluated p53 overexpression by immunohistochemistry in patients without neoplasms whose serum p53 levels were positive. However, all the results of p53 staining were negative (data not shown).

Changes of Serum p53 Ab Levels After Resection of Tumor

To investigate whether changes in serum p53 Ab levels reflect clinical course, we additionally monitored serum p53 Ab levels at several timepoints after surgery in 4 surgically treated patients with CRC whose preoperative serum p53 Abs were positive. In all 4 patients the level of serum p53 Ab had

TABLE 3. Positivity of Serum p53 Abs in Each Study Population

Population	Subject No.	p53 Abs-positive No.	(%)	*P* Value Against HC
HC	63	1	1.6	—
UC	286	43	15.0	0.0014
UC without CRC	278	37	13.3	0.0063
UC with dysplasia	5	2	40.0	<0.0001
UC with CRC	8	6	75.0[a,b]	<0.0001
Sporadic CRC	82	43	52.4	<0.0001

HC, healthy control; UC, ulcerative colitis; CRC, sporadic colorectal cancer.
[a] $P = 0.0002$ against UC without CRC.
[b] $P = 0.2828$ against sporadic CRC.

TABLE 4. Association Between Clinicopathologic Features of UC and Serum p53 Ab Positivity

Characteristics	Patients No.	p53 Abs-positive No.	(%)	*P* Value
Clinical disease activity				
Mild	183	31	16.9	0.390
Moderate	99	12	12.1	
Severe	4	0	0	
Extent of disease				
Proctitis/PS	67	21	11.9	0.744
Left-sided colitis	84	13	15.5	
Pan-colitis	133	8	15.8	
(Others	2	1	50.0	
Extent of disease in patients with duration ≥8 years				
Proctitis/PS	25	2	8.0	0.144
Left-sided colitis	50	9	18.0	
Pan-colitis	68	18	26.5	
Duration of disease				
<8 years	142	13	9.2	0.008
≥8 years	144	30	20.8	
Duration of disease in patients without CRC				
<8 years	140	13	9.2	0.053
≥8 years	138	24	17.4	
Extraintestinal signs				
Positive	21	4	19.0	0.534
Negative	265	39	14.7	

CRC, sporadic colorectal cancer; PS, proctosigmoiditis.
Clinical disease activity was defined by Truelove-Witts criteria.

fallen 4 to 9 months after surgery, and had fallen to within the normal range in 2 patients.

DISCUSSION

There is a need for new biomarkers for early detection, diagnosis, and prognosis to improve the outcome of UC-associated CRCs. In the framework of dysplasia-carcinoma sequence in UC in which mutations in the *p53* gene are the early event,[21] immunohistochemical detection of mutated p53 protein has been widely employed, and its usefulness in CRC surveillance for UC and association of abnormal staining with increased CRC-related mortality in patients with UC were demonstrated.[22,36]

In the present study, serum anti-p53 autoantibody was evaluated as a possible screening tool and we demonstrated that 75.0% of patients with CRC and 40.0% of patients with dysplasia had p53 Abs, while only 12.8% of patients without neoplasia had p53 Abs in the UC population. This is the first report to show a prevalence of serum p53 Abs in UC-associated neoplastic lesions. This indirectly raised the hypothesis that p53 antibodies may be associated with progression to cancer based on the fact that mutation and loss of heterozy-

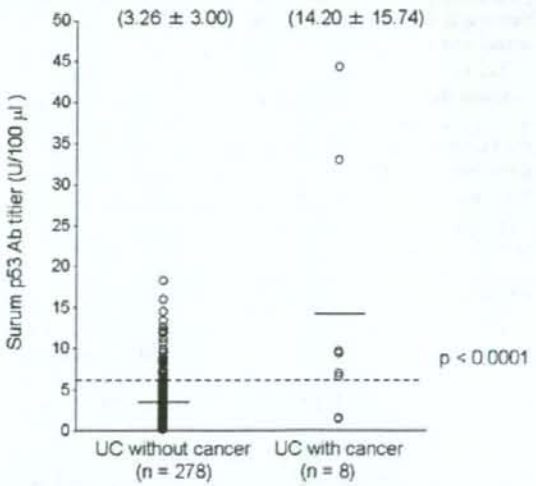

FIGURE 1. Serum p53 Ab titer in ulcerative colitis (UC) patients with colorectal cancer and UC patients without cancer. The p53 Ab levels of patients with cancer ranged from 1.5 U/100 μL to 44.4 U/100 μL, significantly higher than those of patients without cancer ($P < 0.001$).