

INFLAMMATORY BOWEL DISEASE

Possible role of REG I α protein in ulcerative colitis and colitic cancer

A Sekikawa, H Fukui, S Fujii, A Nanakin, N Kanda, Y Uenoyama, T Sawabu, H Hisatsune, T Kusaka, S Ueno, H Nakase, H Seno, T Fujimori, T Chiba



Gut 2005;54:1437–1444. doi: 10.1136/gut.2004.053587

See end of article for authors' affiliations

Correspondence to:
Dr H Fukui, Department of
Gastroenterology and
Hepatology, Kyoto
University Graduate
School of Medicine, 54,
Kawaramachi-shogoin,
Sakyo-ku, Kyoto 606-
8507, Japan; hfukui@
kuhp.kyoto-u.ac.jp

Revised version received
24 March 2005
Accepted for publication
5 May 2005
Published online first
24 May 2005

Background and aims: Although *regenerating gene* (REG) I α protein may be involved in the inflammation and carcinogenesis in the gastrointestinal tract, its pathophysiological role in ulcerative colitis (UC) and the resulting colitic cancer remains unclear. We investigated expression of the *REG I α* gene and its protein in UC and colitic cancer tissues. We examined whether cytokines are responsible for *REG I α* gene expression and whether REG I α protein has a trophic and/or an antiapoptotic effect on colon cancer cells.

Methods: Expression of *REG I α* mRNA and its gene product in UC tissues was analysed by real time reverse transcription-polymerase chain reaction and immunohistochemistry, respectively. The effects of cytokines on *REG I α* promoter activity were examined in LoVo cells by luciferase reporter assay. The effects of REG I α protein on growth and H₂O₂ induced apoptosis were examined in LoVo cells by MTT and TUNEL assays, respectively.

Results: REG I α protein was strongly expressed in inflamed epithelium and in dysplasias and cancerous lesions in UC tissues. The level of *REG I α* mRNA expression in UC tissues correlated significantly with severity of inflammation and disease duration. *REG I α* promoter activity was enhanced by stimulation with interferon γ or interleukin 6. REG I α protein promoted cell growth and conferred resistance to H₂O₂ induced apoptosis in LoVo cells. REG I α protein promoted Akt phosphorylation and enhanced Bcl-xL and Bcl-2 expression in LoVo cells.

Conclusions: The *REG I α* gene is inducible by cytokines and its gene product may function as a mitogenic and/or an antiapoptotic factor in the UC-colitic cancer sequence.

The *regenerating gene* (*Reg*) was originally isolated from a complementary DNA (cDNA) library derived from regenerating rat pancreatic islets, and its human homologue was named *REG I α* .¹ *REG I α* protein is predominantly expressed in the normal pancreas, and at lower levels in the stomach and colon, implying physiological roles for *REG I α* in these organs.^{2,3} Indeed, recent studies have reported that *REG I α* is overexpressed in pancreatitis,^{4,5} *Helicobacter pylori* induced gastritis,⁶ and in gastric ulcer lesions,^{7,8} suggesting that has an important role in the pathogenesis of gastrointestinal inflammatory diseases. Moreover, it is interesting that *REG I α* gene was identified as a distinctly overexpressed gene in inflammatory bowel disease by microarray analyses.^{9,10} However, it is not clear how *REG I α* gene expression is enhanced in such inflammatory conditions.

Although we and others have previously suggested that *Reg* protein has a trophic effect on mammalian epithelial cells,^{11–13} its biological functions are still unclear. Recently, *REG I α* was suggested to be involved not only in inflammatory diseases but also in carcinogenesis in various gastroenterological tissues, such as the stomach,^{14,15} colon,¹⁶ bile duct,¹⁷ and pancreas³; however, its involvement in ulcerative colitis (UC) associated colorectal cancer (colitic cancer) is not known. As the *REG I α* gene is probably overexpressed in UC,^{9,10} *REG I α* may play an important role as a trophic or other factor in the development of colitic cancers. In the present study, in order to elucidate the role of *REG I α* in the UC-colitic cancer sequence, we investigated the relationship between *REG I α* expression and clinicopathological factors in patients with UC and colitic cancer. Furthermore, in *in vitro* studies, we examined whether cytokines enhance *REG I α* gene expression and whether *REG I α* has a trophic and/or an antiapoptotic effect on colon cancer cells.

MATERIALS AND METHODS

Tissue specimens and histological examination

Colon biopsy specimens were obtained by endoscopy from 24 patients with UC (15 men and nine women; mean age 45.6 years (range 19–79); mean disease duration 6.4 years (range 0–19)), four patients with Crohn's disease (two men and two women; mean age 36.0 years (range 26–50); mean disease duration 5.5 years (range 0–15)), eight patients with proctitis (six men and two women; age range 40–79 years), 10 patients with sporadic colon adenoma (seven men and three women; age range 55–85 years), and five normal controls (five men; age range 33–38 years) at Kyoto University Graduate School of Medicine. Tissue specimens were used for real time reverse transcription-polymerase chain reaction (RT-PCR) and histological analyses. This work was done with the approval of the Review Board of Kyoto University Hospital, and informed consent was obtained from all patients.

A total of seven colitic cancer lesions (location: five rectum, one sigmoid, one descending; histology: four well differentiated adenocarcinomas, three mucinous adenocarcinomas) were obtained from surgically resected specimens from four UC patients (two men and two women; age range 44–58 years; disease duration 11–25 years) and eight sporadic

Abbreviations: *Reg*, regenerating gene; IFN, interferon; TNF, tumour necrosis factor; IL, interleukin; CINC, cytokine induced neutrophil chemoattractant; UC, ulcerative colitis; TUNEL, TdT mediated dUTP nick end labelling; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; WST-8, 2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; RT-PCR, reverse transcription-polymerase chain reaction; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFP, enhanced green fluorescent protein gene

colon cancer lesions (location: three rectum, two sigmoid, one descending, one ascending, one caecum; two well differentiated adenocarcinomas, six moderately differentiated adenocarcinomas) were obtained from eight non-UC patients (five men and three women; age range 65–79 years) at Dokkyo University School of Medicine. The colitic cancer tissue specimens were fixed in 10% formalin solution, embedded in paraffin, and subjected to histological analyses. Sporadic colon cancer tissue specimens were used for real time RT-PCR and histological analyses. This work was done with the approval of the Dokkyo University Surgical Pathology Committee, and informed consent was obtained from all patients.

The diagnosis of UC was based on established endoscopic and histological criteria,¹⁸ and the degree of inflammation was evaluated according to Matts' grade¹⁸ throughout the experiments.

Immunohistochemical staining

Immunohistochemical stainings for proliferating cell nuclear antigen (PCNA) and REG α protein were performed, as described previously,^{6,14} using antihuman PCNA antibody (PC10; Dako Japan, Kyoto, Japan; dilution 1:1000) and antihuman REG α antibody (dilution 1:2000).³ A cancerous specimen was considered positive for REG α when more than 20% of the tumour cells were positively stained.¹⁴

Real time RT-PCR

Total RNA was isolated from colonic biopsy samples and seven human colon cancer cell lines (Caco-2, COLO 205, DLD-1, HT-29, LoVo, SW403, and WiDr) with Trizol reagent (Gibco BRL, Rockville, Maryland, USA). Total RNA (5 μ g) was reverse transcribed using oligo-dT primer (Applied Biosystems, Branchburg, New Jersey, USA) and RT product was amplified by PCR, as previously described.^{6,14} TaqMan quantitative real time PCR was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). The following set of primers and probe for human REG α was prepared: human REG α 5'-CTA GAG GCA ACT GGA AAA TAC ATG TCT-3' (sense), 5'-GTT GGA GAG ATG GTC CGG TTT-3' (antisense), and 5'-FAM-AAC GGA GTC AAA AAT T (probe). In addition, a set of primers and probe for human *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) was synthesised by Applied Biosystems.

Each amplification consisted of a 50 μ l reaction mixture with 50 ng of cDNA, 250 nM of REG α probe (or 100 nM of GAPDH probe), 900 nM of REG α primer (or 200 nM of GAPDH primer), and 1 \times TaqMan universal PCR master mixture (Applied Biosystems). PCR cycling conditions were 50°C for two minutes, 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds. A template free negative control was included in all amplifications, and each assay was performed in duplicate. The intensity of the fluorescent dye was determined, and expression levels of REG α mRNA were normalised to GAPDH mRNA expression levels.

Cell culture and treatment

Colon cancer cell lines LoVo and SW403 were routinely maintained in RPMI1640 medium (Invitrogen, Grand Island, New York, USA) with 10% fetal bovine serum (Invitrogen) in a humidified incubator at 37°C with an atmosphere of 5% CO₂. These cell lines were used for assessment of REG α promoter activity by interferon γ (IFN- γ ; Roche, Mannheim, Germany), tumour necrosis factor α (TNF- α ; Roche), interleukin (IL)-1 β (Roche), IL-5 (Pepro Tech Inc, Rocky Hill, New Jersey, USA), IL-6 (Roche), IL-8 (Roche), and IL-13 (Pepro

Tech Inc). In addition, LoVo cells were used for assays of cell growth and apoptosis.

Luciferase activity assay and transfection

The human REG α promoter from -1195 to +78 was generated from human stomach DNA by PCR using the following set of primers containing Mlu I and Bgl II restriction site, respectively: 5'-CTT ACG CGT GAA TTC CTG GGC TCA AGT GA-3' and 5''-CCC GAA GAT TTT AGA TCT ACA GTG C-3''. The cloned nucleotides of the promoter were inserted into the position between Mlu I and Bgl II restriction site, upstream of the luciferase gene in the pGL3-Basic vector (Promega, Madison, Wisconsin, USA), and the construct was named hREG α -Luc.

LoVo and SW403 cells (2 \times 10⁴) were seeded 24 hours before transfection in 12 well plates (Iwaki, Funabashi, Japan). Cells were cotransfected with 700 ng of a hREG α -Luc construct and 7 ng of Renilla luciferase plasmid pRL-TK (as a control of transfection efficiency) in the OptiGen medium (Gibco, Grand Island, New York, USA) using FuGENE 6 transfection reagent (Roche, Indianapolis, Indiana, USA) according to the manufacturer's protocol. Forty eight hours later, cells were stimulated by IFN- γ , TNF- α , IL-1 β , IL-5, IL-6, IL-8, and IL-13 for 3, 6, 12, and 24 hours.

Luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega) according to manufacturer's protocol. Firefly luciferase and Renilla luciferase activities were assayed by luminometer (Lumat LB 9506; Berthold, Germany). Results obtained were normalised for Renilla luciferase activity and expressed as fold of activity of the untreated cell group at the 0 hour time point.

Transfection and expression of the human REG α cDNA

The full length of human REG α cDNA was inserted into the pIRES2-EGFP vector containing cytomegalovirus promoter driving the enhanced green fluorescent protein gene (EGFP) (Clontech, Palo Alto, California, USA). After cloning and verifying the nucleotides of the human REG α cDNA by sequencing, the construct was named pIRES2-hREG α , and pIRES2-EGFP vector without insert was used as a control.

Plasmids were stably transfected into LoVo cells using FuGENE 6 transfection reagent, as described above. To select cells with stable expression of the pIRES2-hREG α and pIRES2-EGFP, cells were cultured for over 3–4 weeks in medium containing G418 (Gibco; 400 μ g/ml) and surviving colonies were pooled.

Preparation of conditioned medium

To prepare conditioned medium, we cultured human embryonic kidney (HEK) 293T cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. According to the manufacturer's protocol, cells were transfected with 10 μ g of pIRES2-hREG α or control plasmid using FuGENE 6 transfection reagent. The medium was replaced by serum free RPMI1640 medium after a 48 hour incubation period. The conditioned medium was then collected and stored frozen as a source of recombinant REG α protein.

Cell growth assay

Cell growth was assessed by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). LoVo cells (5 \times 10³), stably transfected with pIRES2-hREG α (LoVo-REG α cells) or pIRES2-EGFP (LoVo-EGFP) vector, were plated in 96 well microplates. Cells were incubated in serum free RPMI1640 medium for 24, 48, and 72 hours. After addition of 10 μ l of the Cell Counting Kit-8 reagent followed by a three hour incubation, the plates were read at 450 nm in a spectrophotometer (Molecular

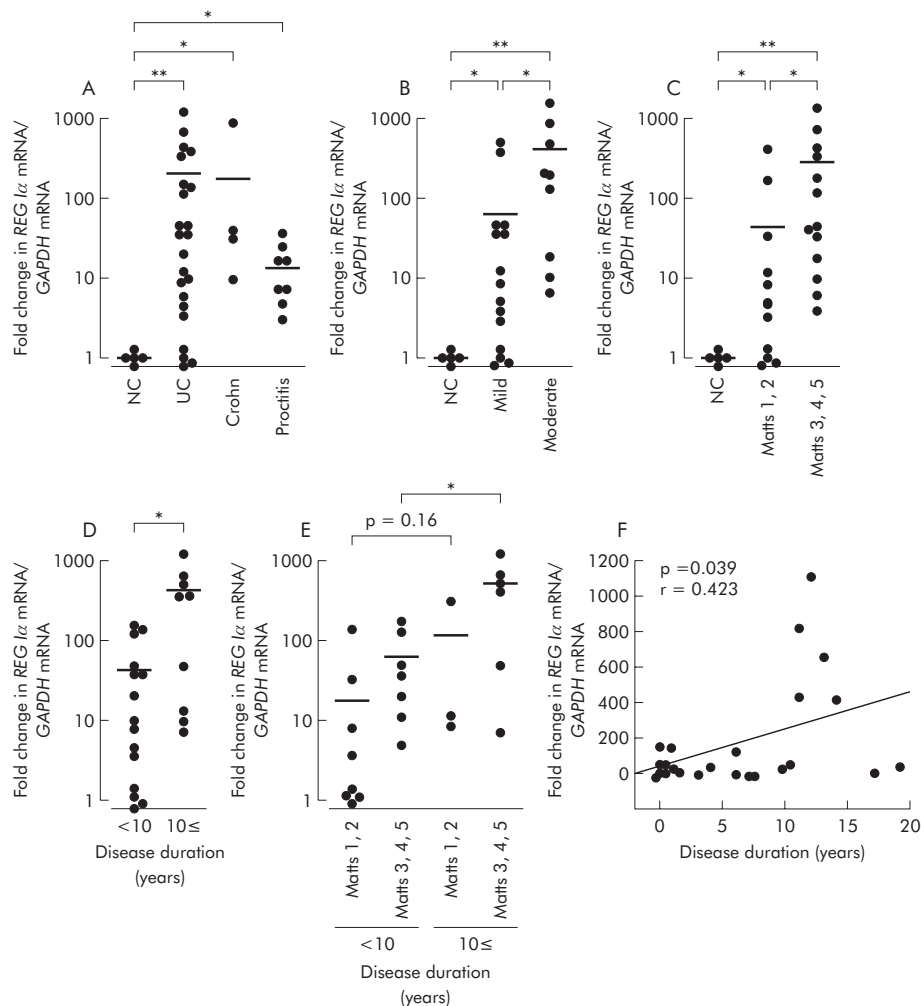


Figure 1 *REG I α* mRNA expression in ulcerative colitis tissues. (A) *REG I α* mRNA expression levels in normal colon (NC), ulcerative colitis (UC), Crohn's colitis, and proctitis tissues. Comparison of *REG I α* mRNA expression levels among groups subdivided by endoscopic appearance (B), histological findings (C), or disease duration (D, E). (F) Correlation between *REG I α* expression levels and disease duration. All results are expressed as fold change in *REG I α* mRNA/*GAPDH* mRNA ratio relative to the normal control group. Significant difference between two groups: * $p < 0.05$, ** $p < 0.01$. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Devices Co., Sunnyvale, California, USA). To assess the effect of REG I α in the medium, we added anti-REG I α antibody (50 $\mu\text{g/ml}$) to serum free RPMI1640 medium,^{13, 19} and cells incubated for 72 hours were also evaluated in the same procedure.

Cell growth assay was also performed to examine the trophic effect of REG I α protein on colon cancer cells. Briefly, LoVo cells (5×10^3) were cultured in 96 well microplates for 24 hours. Then, the medium was changed to conditioned medium containing human recombinant REG I α or control medium, and cells were incubated for 24, 48, and 72 hours. To assess the effect of REG I α in the medium, we added anti-REG I α antibody to control or REG I α containing medium, and cells incubated for 72 hours were also evaluated in the same procedure.

TUNEL assay

LoVo-REG I α cells (1×10^4) and control LoVo-EGFP cells were cultured in four well culture slides (Falcon, Bedford, Massachusetts, USA). Twenty four hours later, cells were incubated for two hours with different concentrations (0–10 mmol/l) of H_2O_2 in serum free medium. Thereafter, cells were incubated in routine medium for 24 hours. After wash with phosphate buffered saline, slides were fixed with 10%

buffered formalin for 15 minutes and then stained by In Situ Cell Death Detection Kit (Roche) according to the supplied protocol. TdT mediated dUTP nick end labelling (TUNEL) index was calculated as the percentage of positive cells. To assess the effect of REG I α in the medium, we added anti-REG I α antibody (50 $\mu\text{g/ml}$) to routine medium immediately after treatment with H_2O_2 (5 mmol/l).

TUNEL assay was also performed to examine the anti-apoptotic effect of REG I α protein on colon cancer cells. LoVo cells (5×10^3) were cultured in four well culture slides for 24 hours, followed by a 24 hour incubation with conditioned medium containing human recombinant REG I α or control medium. Then, cells were incubated for another two hours with different concentrations (0–10 mmol/l) of H_2O_2 , and subjected to TUNEL assay 24 hours later. To assess the effect of REG I α in the medium, we added anti-REG I α antibody to control or REG I α containing medium immediately after treatment with H_2O_2 (5 mmol/l).

Detection of phosphorylated, non-phosphorylated Akt, and Bcl family proteins

LoVo cells were cultured in 10 cm dishes for 24 hours. After washing with phosphate buffered saline, the medium was changed to conditioned medium containing human

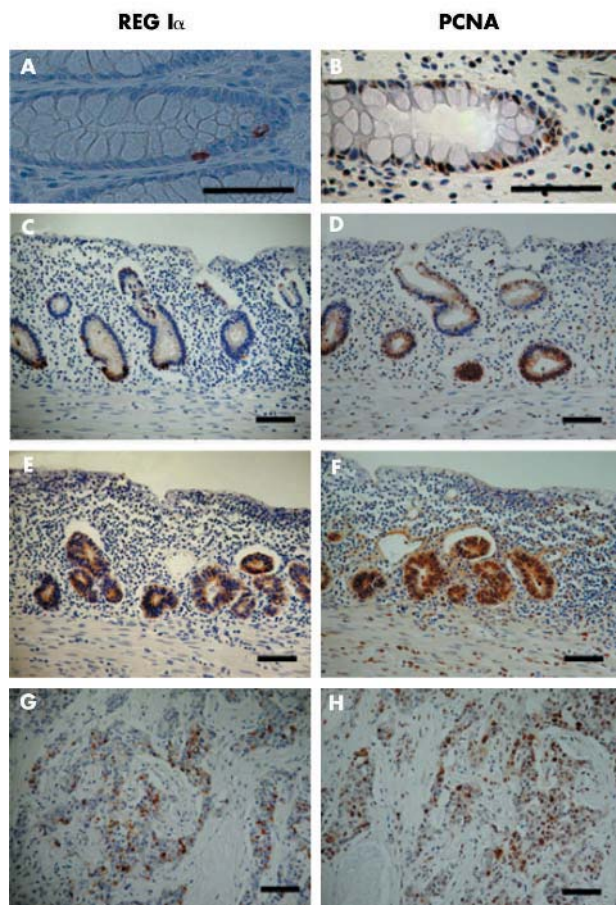


Figure 2 Immunostaining for REG $I\alpha$ protein and proliferating cell nuclear antigen (PCNA) in normal colon (A, B), ulcerative colitis (C, D), dysplasia (E, F), and colitic cancer (G, H) tissues. REG $I\alpha$: A, C, E, G; PCNA: B, D, F, H. Bars = 100 μ m.

recombinant REG $I\alpha$ or control medium, and cells were incubated for another 12 hours. Cells were then mixed with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (NP-40), 50 mM NaF, and 1 \times proteinase inhibitor (Complete Mini; Roche). Protein extract (10 μ g) was fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. After transfer to a polyvinylidene difluoride membrane, western blots were performed using anti-Akt, antiphospho specific Akt (Ser473) (New England Biolabs, Beverly, Massachusetts, USA), anti-Bcl-2, anti-Bcl-xL, anti-Mcl-1 (BD Sciences, San Jose, California, USA), and anti- β -actin antibodies (Sigma Chemical Co., St Louis, Missouri, USA), as previously reported.²⁰

Statistical analysis

All values are expressed as mean (SEM). Statistical differences between the two groups were assessed by the unpaired two tailed *t* test or by the Mann-Whitney U test when data were not parametric. The relationship between REG $I\alpha$ mRNA level and disease duration was assessed by linear regression analysis. A *p* value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Expression of REG $I\alpha$ mRNA in normal colonic and ulcerative colitis mucosa

REG $I\alpha$ mRNA expression was detectable by real time RT-PCR in all colonic mucosa from both control and UC patients. As

shown in fig 1A, the level of REG $I\alpha$ mRNA expression was significantly greater in UC tissues than in normal colonic tissues. Furthermore, levels of REG $I\alpha$ mRNA expression in Crohn's colitis and proctitis tissues were also significantly greater than that in normal colonic tissues. We next analysed the relationship between REG $I\alpha$ mRNA expression and several clinicopathological factors, and found that REG $I\alpha$ mRNA expression was significantly enhanced as the severity of endoscopic or histological features increased (fig 1B, 1C). Moreover, REG $I\alpha$ mRNA expression in UC patients of long duration (≥ 10 years) was significantly greater than those of short duration (< 10 years) (fig 1D). We further subdivided patients by both disease duration and histological findings and analysed REG $I\alpha$ mRNA expression in each group. As shown in fig 1E, in high-Matts score group REG $I\alpha$ gene expression level in patients with long duration UC was significantly greater than that in patients with short duration UC. In addition, in low-Matts score group REG $I\alpha$ gene expression level in patients with long duration was also greater than in patients with short duration although this was not statistically significant ($p = 0.16$). REG $I\alpha$ mRNA expression in UC tissues was significantly correlated with disease duration (fig 1F) but not with age or sex (data not shown).

Expression of REG $I\alpha$ protein and PCNA in normal colonic and ulcerative colitis mucosa, and in dysplasia and colitic cancer

In normal colonic mucosa, REG $I\alpha$ immunoreactivity was detected only in a few epithelial cells in the basal portion of crypts (fig 2A). Colonic epithelial cells positive for PCNA were also observed in the basal portion of crypts (fig 2B).

In UC mucosa, both the number of REG $I\alpha$ positive cells in crypts and the intensity of REG $I\alpha$ immunoreactivity were increased. Strongly REG $I\alpha$ positive cells were mainly observed in the lower part of the colonic mucosa (fig 2C). The number of PCNA positive cells in crypts was also increased, and the distribution of such cells was similar to that of REG $I\alpha$ positive cells (fig 2D).

REG $I\alpha$ immunoreactivity was detected in the cytoplasm of not only dysplastic but also colitic cancer cells (fig 2E, 2G). REG $I\alpha$ expression was detected in all seven of the colitic cancer lesions, originating from four patients examined. PCNA was also strongly expressed in both dysplastic and cancerous cells in all colitic cancer tissues examined (fig 2F, 2H).

Expression of REG $I\alpha$ mRNA in colon cancer cell lines, sporadic colon adenomas, and cancers

Expression of REG $I\alpha$ mRNA was detected in all seven colon cancer cell lines examined using the RT-PCR method (fig 3). Then, we examined REG $I\alpha$ mRNA expression in colon adenomas, cancers, and their neighbouring normal colon mucosa by real time RT-PCR. REG $I\alpha$ mRNA expression level in colon adenomas ($n = 10$; 159 (51)) was significantly higher than that in normal colon mucosa ($n = 10$; 1.0 (0.2))

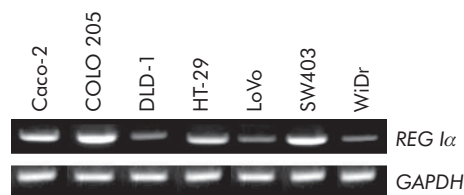


Figure 3 Detection of REG $I\alpha$ mRNA in various human colon cancer cell lines by reverse transcription-polymerase chain reaction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

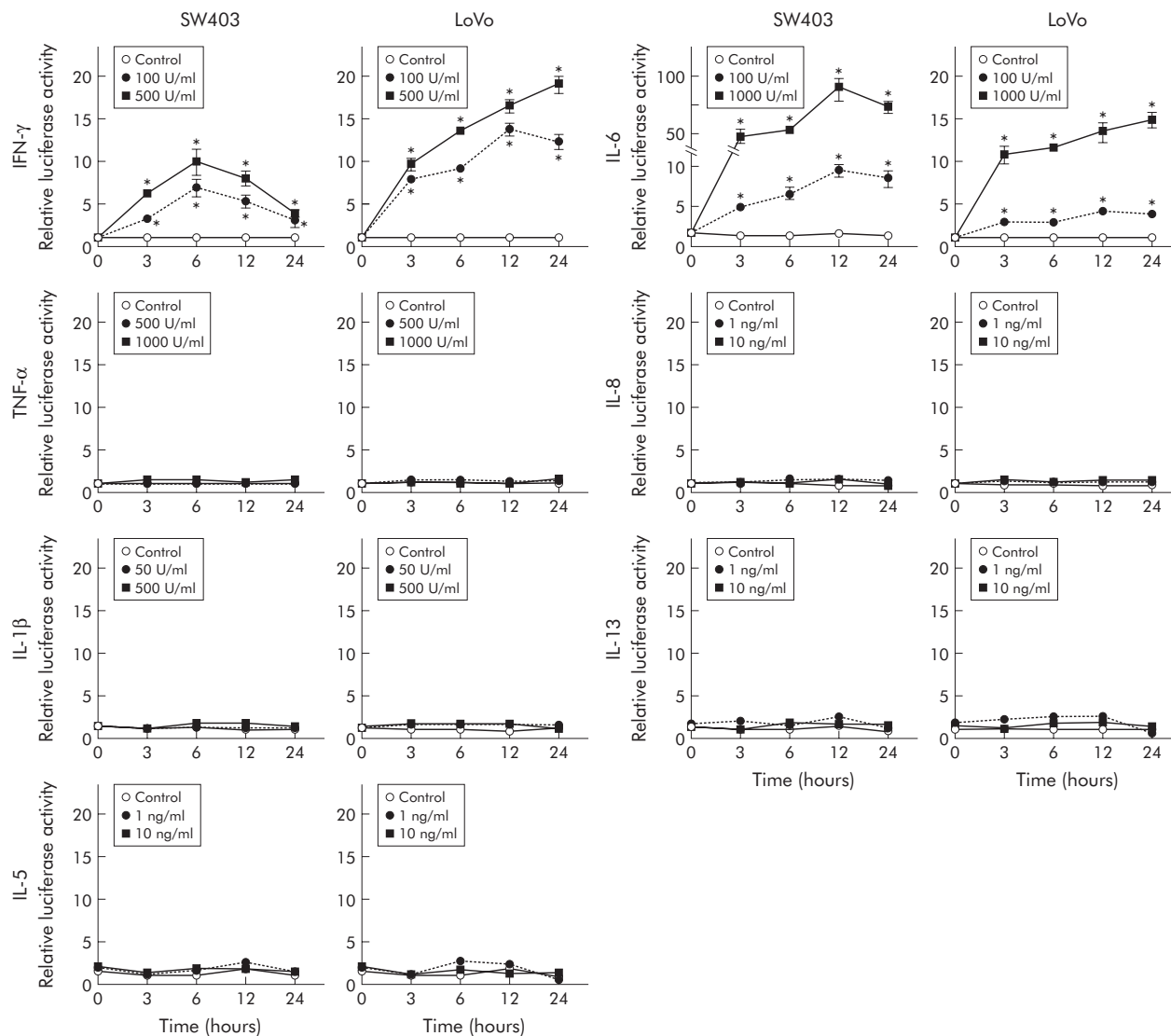


Figure 4 *REG* α promoter activity in LoVo and SW403 cells in response to various cytokines. Cells were cotransfected with hREG α (-1195/+78)-Luc construct and Renilla luciferase plasmid pRL-TK (as a control for transfection efficiency). Forty eight hours later, cells were stimulated with interferon γ (IFN- γ), tumour necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-5, IL-6, IL-8, or IL-13. Luciferase activity was measured in extracts from transfected LoVo and SW403 cells and normalised to Renilla luciferase activity. All results are expressed as fold of the activity of the untreated cell group at the 0 hour time point. Vertical lines represent means (SEM) of four independent experiments. * p <0.05 versus control at the same time point.

(p <0.05). Moreover, the *REG* α mRNA expression level in colon cancers (n =8; 1260 (710)) was significantly higher than that in normal colon mucosa (p <0.05).

Effects of cytokines on *REG* α promoter activity in colon cancer cell lines

The effect of several cytokines on human *REG* α promoter activity was analysed by transient expression assays in two human colon cancer cell lines (fig 4). In both LoVo and SW403 cells transfected with the hREG α -Luc construct, luciferase activity was significantly elevated at three hours after stimulation with IFN- γ (100 and 500 U/ml) and this elevation was sustained for 24 hours. Similarly, human *REG* α promoter activity was enhanced in both cell lines at three hours after treatment with IL-6 (100 and 1000 IU/ml) and again the elevation was sustained for 24 hours. In contrast, treatment with TNF- α , IL-1 β , IL-5, IL-8, or IL-13 did not affect luciferase activity in the two cell lines.

Effect of *REG* α on cell growth in LoVo cells

LoVo cells transfected with pIRES2-hREG α (LoVo-REG α) showed significantly higher WST-8 cleavage levels than LoVo cells transfected with pIRES2-EGFP (LoVo-EGFP; control) at each time point (24–72 hours) of incubation (fig 5A). Increased WST-8 cleavage in the LoVo-REG α cell group was suppressed almost to control levels by addition of anti-REG α antibody (fig 5B), suggesting that enhanced cell growth in LoVo-REG α cells is caused by secreted REG α protein.

REG α conditioned medium significantly increased WST-8 cleavage in LoVo cells after 24–72 hours of incubation time (fig 5D). As shown in fig 5E, the increased WST-8 cleavage in REG α -treated LoVo cells was abolished by concomitant administration of anti-REG α antibody.

Antiapoptotic effect of *REG* α in LoVo cells

The LoVo-REG α cell group showed significantly lower TUNEL positivity than the LoVo-EGFP group when they were

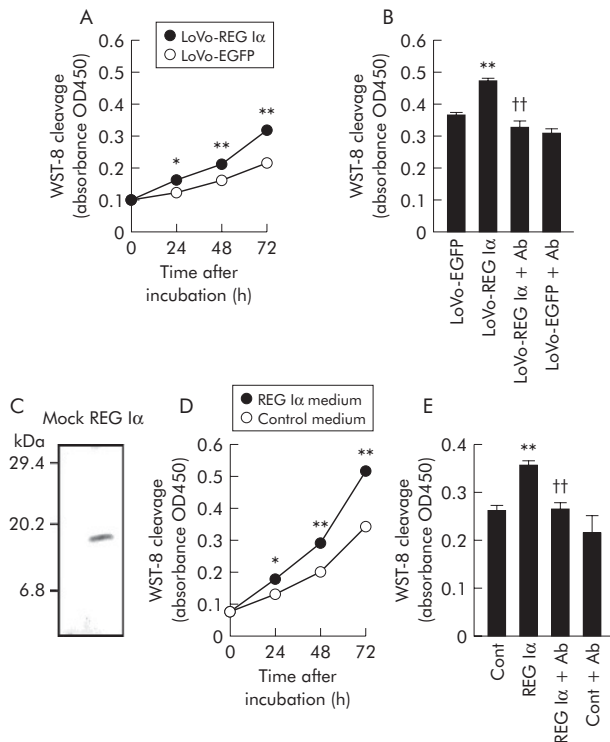


Figure 5 Effects of *REG I α* gene induction or *REG I α* conditioned medium on the growth of human colon cancer cells. (A) Effect of *REG I α* gene induction on growth of human colon cancer cells. LoVo cells transfected with pIRES2-h*REG I α* (LoVo-*REG I α*) or pIRES2-EGFP (LoVo-EGFP; control) plasmids were cultured for 24, 48, and 72 hours. WST-8 cleavage in each group was measured by ELISA (absorbance OD450), as described in materials and methods. (B) Effect of anti-*REG I α* antibody on cell growth of LoVo-*REG I α* and LoVo-EGFP. LoVo-*REG I α* and LoVo-EGFP cells were incubated with or without anti-*REG I α* antibody (Ab, 50 μ g/ml) for 72 hours. (C) Source of *REG I α* protein. Human embryonic kidney HEK293T cells were transfected with a human *REG I α* cDNA expression plasmid or a control plasmid, and the medium conditioned by these cells was collected. Release of *REG I α* protein into the conditioned medium of human *REG I α* cDNA transfected cells was confirmed by western blot analysis with an anti-human *REG I α* monoclonal antibody. (D) Time course of the effect of *REG I α* conditioned medium on growth of human colon cancer cells. LoVo cells were incubated with *REG I α* or control medium for 24–72 hours. (E) Effect of anti-*REG I α* antibody on growth of colon cancer cells promoted by *REG I α* protein. Anti-*REG I α* antibody (50 μ g/ml) was added to control or *REG I α* medium, followed by incubation for 72 hours. All results are expressed as the mean (SEM) of four samples. * p <0.05, ** p <0.01 versus control (LoVo-EGFP cells (A, B) or control medium treated cells (D, E)) group at the same time point. ††Significantly lower than LoVo-*REG I α* cells (B) or *REG I α* medium treated cells (E) (p <0.01).

treated with H_2O_2 at concentrations of 2.5–10 mM, suggesting that *REG I α* overexpressing cells are more resistant to apoptosis induced by H_2O_2 (fig 6A). The decreased TUNEL positivity in the LoVo-*REG I α* cell group was significantly reversed by treatment with anti-*REG I α* antibody (fig 6B), suggesting that secreted *REG I α* protein confers an anti-apoptotic effect on colon cancer cells.

LoVo cells treated with *REG I α* conditioned medium showed a significantly lower TUNEL positivity than cells treated with control medium when they were exposed to H_2O_2 at concentrations of 2.5–10 mM (fig 6C). The decreased TUNEL positivity in *REG I α* treated LoVo cells was significantly reversed by concomitant administration of anti-*REG I α* antibody (fig 6D).

Treatment with *REG I α* conditioned medium enhanced the phosphorylation of Akt in LoVo cells (fig 7A). Moreover, *REG*

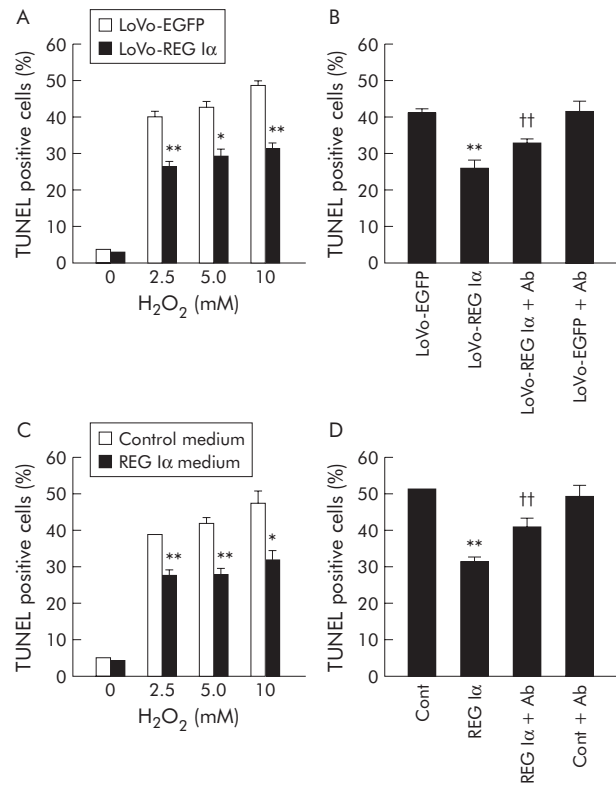


Figure 6 Effects of *REG I α* gene induction or *REG I α* conditioned medium on H_2O_2 induced apoptosis of human colon cancer cells. (A) Resistance to H_2O_2 induced apoptosis in human colon cancer cells transfected with *REG I α* cDNA. LoVo cells transfected with pIRES2-h*REG I α* (LoVo-*REG I α*) or pIRES2-EGFP (LoVo-EGFP; control) plasmids were treated with H_2O_2 (0–10 mM) for two hours, followed by incubation in fresh culture medium for 24 hours. The percentage of TUNEL positive cells was evaluated, as described in materials and methods. (B) Effect of anti-*REG I α* antibody (Ab, 50 μ g/ml) on H_2O_2 (5 mM) induced apoptosis in LoVo-*REG I α* and LoVo-EGFP cells. (C) Resistance to H_2O_2 induced apoptosis in human colon cancer cells treated with *REG I α* conditioned medium. LoVo cells were incubated with conditioned medium containing recombinant human *REG I α* or control medium for 24 hours, followed by incubation for two hours with medium containing H_2O_2 (0–10 mM). (D) Effect of anti-*REG I α* antibody on H_2O_2 (5 mM) induced apoptosis in LoVo cells. Anti-*REG I α* antibody (50 μ g/ml) was added to control or *REG I α* medium. All results are expressed as the mean (SEM) of four samples. * p <0.05, ** p <0.01 versus control (LoVo-EGFP cells (A, B) or control medium treated cells (C, D)) at the same dose point. ††Significantly higher than LoVo-*REG I α* cells (B) or *REG I α* medium treated cells (D) (p <0.01).

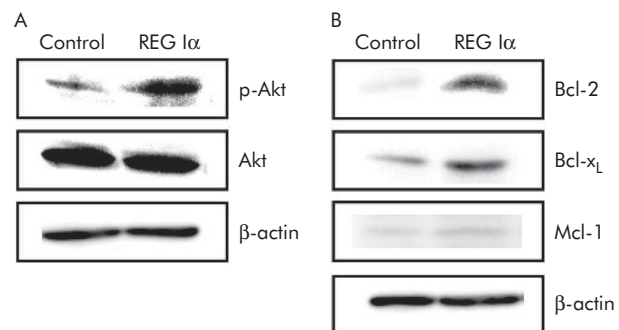


Figure 7 Effects of *REG I α* conditioned medium on phosphorylation of Akt and expression of Bcl family proteins in colon cancer cells. Western blotting indicated that phosphorylation of Akt (A) and expression of Bcl-2 and Bcl-xL but not Mcl-1 (B) were enhanced in LoVo cells after 12 hours of incubation with *REG I α* conditioned medium.

I α conditioned medium clearly increased Bcl-2 and Bcl-xL expression in LoVo cells whereas REG I α did not affect Mcl-1 expression (fig 7B).

DISCUSSION

In the present study, we have shown that REG I α is expressed in only a few small cells in the mid to basal portion of a colonic crypt where putative stem cells reside and continuous cell renewal is occurring.^{21, 22} Interestingly, in the gastric epithelium, REG I α is expressed not only in enterochromaffin-like and chief cells but also in immature small cells in the proliferating neck zone of gastric glands.^{14, 23} As REG I α protein is indeed mitogenic to gastrointestinal epithelial cells,¹¹ REG I α produced in the proliferative zone may play a role in the self renewal of gastrointestinal epithelium under certain physiological conditions.

It is noteworthy that several investigators have identified REG I α as one of the most abundantly expressed genes in the colonic mucosa of patients with UC by gene chip analyses,^{9, 10} even though only a few REG I α positive cells are present in normal colonic mucosa. Confirming these data, we demonstrated in this study that levels of both REG I α gene and its protein were significantly increased in UC mucosa. Furthermore, we also showed that enhancement of REG I α gene expression in the colonic mucosa was correlated with severity of inflammation in UC, as assessed both endoscopically and histologically. These data strongly suggest that REG I α is important in the inflammatory process of UC.

What factors could be responsible for the increase in REG I α expression in UC mucosa? As various cytokines play important roles in the inflammation of UC, we examined in this study whether those cytokines enhance transcription of the REG I α gene in vitro, and found that both IFN- γ and IL-6 significantly stimulated REG I α promoter activity in colon cancer cells. In contrast, IL-8, IL-1 β , TNF- α , IL-5, or IL-13 could not enhance REG I α promoter activity in colon cancer cells. Previous studies have shown that, in addition to IFN- γ and IL-6, TNF- α and CINC-2 β , a mouse homologue of IL-8, can enhance REG I α gene expression in rat pancreatic acinar cells^{19, 24} and in rat gastric mucosa in vivo.²⁵ The discrepancies between these results and our present data may be due to the use of different species, cell types, or experimental conditions. It is well known that expression of both IL-6 and IFN- γ is prominently increased in UC mucosa.²⁶⁻²⁸ Thus although we did not measure levels of those cytokines in this study, it appears reasonable to suppose that increased levels of IL-6 and IFN- γ in the mucosa of UC are at least in part responsible for the enhanced expression of the REG I α gene and its product. It may be noted that although UC is known as a Th2 dominant disease,^{29, 30} none of Th2 cytokines tested in this study had any effect on REG I α promoter activity. These data may suggest that the enhanced REG I α expression in UC mucosa is not a Th2 specific phenomenon but rather a reflection of a general inflammatory condition where IL-6 and IFN- γ expression are elevated.

It is important to clarify whether REG I α is involved in the carcinogenesis of UC associated colorectal cancer. Interestingly, we found in this study that REG I α was expressed not only in the epithelial cells of UC mucosa but also in epithelial cells of dysplastic lesions and in colitic cancer cells. Moreover, we have clearly shown that REG I α expression is significantly increased in patients with long-standing colitis that presents a high risk of colitic cancer.^{31, 32} These findings strongly suggest involvement of REG I α in the UC-colitic cancer sequence. During UC, colonic epithelial cells are continuously injured by inflammation that may induce sustained regeneration of epithelial cells. In the present study, we have shown that REG I α protein has not only growth promoting but also antiapoptotic actions on colonic

cells, and moreover that REG I α protein exerts its antiapoptotic effect at least in part by activating Akt signalling and enhancing Bcl-xL and Bcl-2 expression. Thus REG I α protein induced by UC may play a role in protecting colonic epithelial cells from apoptosis. Conversely, however, as REG I α expression is sustained at high levels in chronic UC, its antiapoptotic action as well as its growth promoting effects may contribute to the development of colitic cancer from UC tissues. Of note, we also found that the distribution of REG I α positive cells was similar to that of PCNA positive cells in colitic cancer tissues as well as in dysplastic and colitis mucosa. Therefore, REG I α produced in epithelial cells as well as in cancer cells may exert a direct action on epithelial cells in colitis, and also on cancer cells themselves. In support of this view, addition of anti-REG I α antibody to the incubation medium abolished not only the growth promoting effect of REG I α conditioned medium but also the enhanced growth of REG I α transfected cells. Similarly, anti-REG I α antibody blocked the antiapoptotic effect of REG I α conditioned medium and abrogated the reduction in apoptosis in REG I α transfected cells. These data strongly suggest that REG I α secreted from epithelial cells or cancer cells exerts growth promoting and antiapoptotic actions in a paracrine or autocrine fashion. On the other hand, it may be noted in this study that colitic cancers develop in only a limited number of patients with UC, although REG I α is ubiquitously expressed in the inflamed UC mucosa. However, it may be emphasised that carcinogenesis is a complicated event in which many factors are involved. Thus REG I α may play a role in the UC-colitic cancer sequence as one of the growth promoting as well as antiapoptotic factors, and function in colitic cancer development not as a tumour initiator but as a tumour promoter.

In the present study, we have shown that REG I α gene expression is increased not only in UC mucosa and colitic cancers but also in sporadic colon adenomas or cancers, using real time RT-PCR analysis, although its expression is not always detected in sporadic colon cancers by northern blot analysis.^{16, 33} In addition, REG I α gene expression was detected in all seven colon cancer cell lines examined. These findings may suggest that REG I α is involved not only in the UC-colitic cancer sequence but also in the adenoma-carcinoma sequence, although the mechanism of carcinogenesis in UC associated colorectal cancer is believed to be distinct from that in sporadic colorectal cancer.^{34, 35} The role of REG I α in the adenoma-carcinoma sequence should also be clarified in future studies.

In summary, we have shown that REG I α is expressed not only in epithelial cells of UC mucosa but also in precancerous dysplastic epithelial cells and colitic cancer cells. The promoter activity of the REG I α gene is stimulated by IFN- γ and IL-6. Moreover, REG I α protein has both mitogenic and antiapoptotic effects on human colon cancer cells in vitro. Together, these results suggest that cytokine induced REG I α in the colonic mucosa plays an important role in the development of UC associated colorectal cancer.

ACKNOWLEDGEMENTS

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We are grateful to Dr Hiroshi Okamoto in Tohoku University Graduate School of Medicine, Sendai, Japan, for providing anti-REG I α antibody.

Authors' affiliations

A Sekikawa, H Fukui, A Nanakin, N Kanda, Y Uenoyama, T Sawabu, H Hisatsune, T Kusaka, S Ueno, H Nakase, H Seno, T Chiba, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan

S Fujii, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan, and Department of Pathology, Dokkyo University School of Medicine, Tochigi, Japan
T Fujimori, Department of Pathology, Dokkyo University School of Medicine, Tochigi, Japan

Conflict of interest: None declared.

REFERENCES

- 1 **Terazono K**, Yamamoto H, Takasawa S, et al. A novel gene activated in regenerating islets. *J Biol Chem* 1988;**263**:2111–14.
- 2 **Watanabe T**, Yonekura H, Terazono K, et al. Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues. *J Biol Chem* 1990;**265**:7432–9.
- 3 **Kimura N**, Yonekura H, Okamoto H, et al. Expression of human regenerating gene mRNA and its product in normal and neoplastic human pancreas. *Cancer* 1992;**70**:1857–63.
- 4 **Satomura Y**, Sawabu N, Mouri I, et al. Measurement of serum PSP/reg-protein concentration in various diseases with a newly developed enzyme-linked immunosorbent assay. *J Gastroenterol* 1995;**30**:643–50.
- 5 **Graf R**, Schiesser M, Lüssi A, et al. Coordinate regulation of secretory stress proteins (PSP/reg, PAP I, PAP II, and PAP III) in the rat exocrine pancreas during experimental acute pancreatitis. *J Surg Res* 2002;**105**:136–44.
- 6 **Fukui H**, Franceschi F, Penland RL, et al. Effects of *Helicobacter pylori* infection on the link between regenerating gene expression and serum gastrin levels in Mongolian gerbils. *Lab Invest* 2003;**83**:1777–86.
- 7 **Asahara M**, Mushiaki S, Shimada S, et al. Reg gene expression is increased in rat gastric enterochromaffin-like cells following water-immersion stress. *Gastroenterology* 1996;**111**:45–55.
- 8 **Kawanami C**, Fukui H, Kinoshita Y, et al. Regenerating gene expression in normal gastric mucosa and indomethacin-induced mucosal lesions of the rat. *J Gastroenterol* 1997;**32**:12–18.
- 9 **Lawrance IC**, Fiocchi C, Chakravarti S. Ulcerative colitis and Crohn's disease: distinctive gene expression profiles and novel susceptibility candidate genes. *Hum Mol Genet* 2001;**10**:445–56.
- 10 **Dieckgraefe BK**, Stenson WF, Korzenik JR, et al. Analysis of mucosal gene expression in inflammatory bowel disease by parallel oligonucleotide arrays. *Physiol Genomics* 2000;**4**:1–11.
- 11 **Fukui H**, Kinoshita Y, Maekawa T, et al. Regenerating gene protein may mediate gastric mucosal proliferation induced by hypergastrinemia in rats. *Gastroenterology* 1998;**115**:1483–93.
- 12 **Watanabe T**, Yonemura Y, Yonekura H, et al. Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg production. *Proc Natl Acad Sci U S A* 1994;**91**:3589–92.
- 13 **Kadowaki Y**, Ishihara S, Miyaoka Y, et al. Reg protein is overexpressed in gastric cancer cells, where it activates a signal transduction pathway that converges on ERK1/2 to stimulate growth. *FEBS Lett* 2002;**530**:59–64.
- 14 **Fukui H**, Fujii S, Takeda J, et al. Expression of Reg Ix protein in human gastric cancers. *Digestion* 2004;**69**:177–84.
- 15 **Dahr DK**, Udagawa J, Ishihara S, et al. Expression of regenerating gene I in gastric adenocarcinomas. *Cancer* 2004;**100**:1130–6.
- 16 **Recherche H**, Montalto G, Mallo GV, et al. pap, reg Ix and reg Iβ mRNAs are concomitantly up-regulated during human colorectal carcinogenesis. *Int J Cancer* 1999;**81**:688–94.
- 17 **Harada K**, Zen Y, Kanemori Y, et al. Human REG I gene is up-regulated in intrahepatic cholangiocarcinoma and its precursor lesions. *Hepatology* 2001;**33**:1036–42.
- 18 **Matts SG**. The value of rectal biopsy in the diagnosis of ulcerative colitis. *Q J Med* 1961;**120**:393–407.
- 19 **Akiyama T**, Takasawa S, Nata K, et al. Activation of Reg gene, a gene for insulin-producing β-cell regeneration: Poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribose)ylation. *Proc Natl Acad Sci U S A* 2001;**98**:48–53.
- 20 **Kanda N**, Seno H, Konda Y, et al. STAT3 is constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. *Oncogene* 2004;**23**:4921–9.
- 21 **Chang WWL**, Nadler NJ. Renewal of the epithelium in the descending colon of the mouse. Cell population kinetics of vacuolated-columnar cells. *Am J Anat* 1975;**144**:39–58.
- 22 **Appleton DR**, Sunter JP, deRodriguez MSB. Cell proliferation in the mouse large bowel, with detail of the analysis of experimental data. In: Appleton DR, eds. *Cell proliferation in the gastrointestinal tract*. London: Pitman, 1980:141–54.
- 23 **Higham AD**, Bishop LA, Dimaline R, et al. Mutations of Reg Ix are associated with enterochromaffin-like cells tumor development in patients with hypergastrinemia. *Gastroenterology* 1999;**116**:1310–81.
- 24 **Duseti NJ**, Mallo GV, Ortiz EM, et al. Induction of lithostathine/reg mRNA expression by serum from rats with acute pancreatitis and cytokines in pancreatic acinar AR-42J cells. *Arch Biochem Biophys* 1996;**330**:129–32.
- 25 **Kazumori H**, Ishihara S, Hoshino E, et al. Neutrophil chemoattractant 2β regulates expression of the Reg gene in injured gastric mucosa in rats. *Gastroenterology* 2000;**119**:1610–22.
- 26 **Kusugami K**, Fukatsu A, Tanimoto M, et al. Elevation of interleukin-6 in inflammatory bowel disease is macrophage- and epithelial cell-dependent. *Dig Dis Sci* 1995;**40**:949–59.
- 27 **Indaram AVK**, Visvalingam V, Locke M, et al. Mucosal cytokine production in radiation-induced proctosigmoiditis compared with inflammatory bowel disease. *Am J Gastroenterol* 2000;**95**:1221–5.
- 28 **Autschbach F**, Giese T, Gassler N, et al. Cytokine/chemokine messenger-RNA expression profiles in ulcerative colitis and Crohn's disease. *Virchows Arch* 2002;**441**:500–13.
- 29 **Fuss IJ**, Heller F, Boirivant M, et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* 2004;**113**:1490–7.
- 30 **Sato T**, Kanai T, Watanabe M, et al. Hyperexpression of inducible costimulator and its contribution on lamina propria T cells in inflammatory bowel disease. *Gastroenterology* 2004;**126**:829–39.
- 31 **Gyde SN**, Prior P, Allan RN, et al. Colorectal cancer in ulcerative colitis: a cohort study of primary referrals from three centres. *Gut* 1988;**29**:206–17.
- 32 **Eaden JA**, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001;**48**:526–35.
- 33 **Macadam RCA**, Sarela AI, Farmery SM, et al. Death from early colorectal cancer is predicted by the presence of transcripts of the REG gene family. *Br J Cancer* 2000;**83**:188–95.
- 34 **Brennall TA**, Crispin DA, Rabinovitch PS, et al. Mutations in the p53 gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology* 1994;**107**:369–78.
- 35 **Lashner BA**, Shapiro BD, Husain A, et al. Evaluation of the usefulness of testing for p53 mutations in colorectal cancer surveillance for ulcerative colitis. *Am J Gastroenterol* 1999;**94**:456–62.

bmjupdates+

bmjupdates+ is a unique and free alerting service, designed to keep you up to date with the medical literature that is truly important to your practice.

bmjupdates+ will alert you to important new research and will provide you with the best new evidence concerning important advances in health care, tailored to your medical interests and time demands.

Where does the information come from?

bmjupdates+ applies an expert critical appraisal filter to over 100 top medical journals. A panel of over 2000 physicians find the few 'must read' studies for each area of clinical interest.

Sign up to receive your tailored email alerts, searching access and more...

www.bmjupdates.com