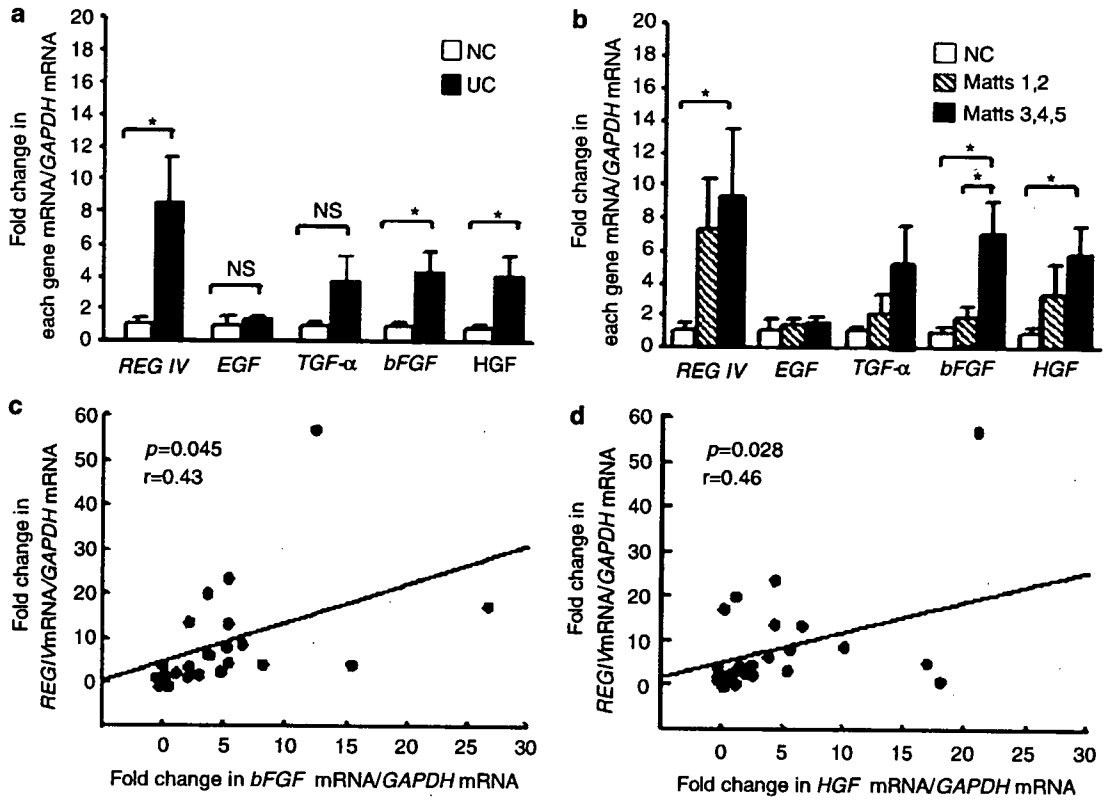


**Figure 3** *REG IV* mRNA expression in UC tissues. (**a**) Expression levels of mRNA for *REG IV* and other *growth factors* in normal colon and UC tissues. (**b**) Comparison of mRNA expression levels for *REG IV* and other *growth factors* among UC groups subdivided by histological findings. Correlation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

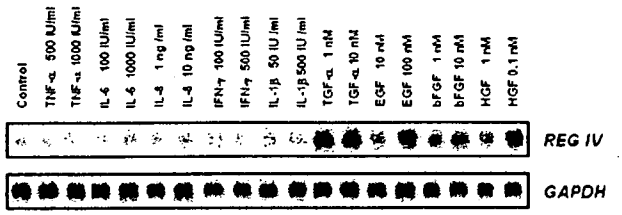


**Figure 4** Effects of various cytokines and growth factors on *REG IV* mRNA expression in SW403 cells. Total RNA (20 $\mu$g) was extracted 12 h after stimulation with cytokines or growth factors and analyzed by Northern blotting using $^{32}$P-labeled cDNAs for *REG IV* and *GAPDH* mRNA.

## 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

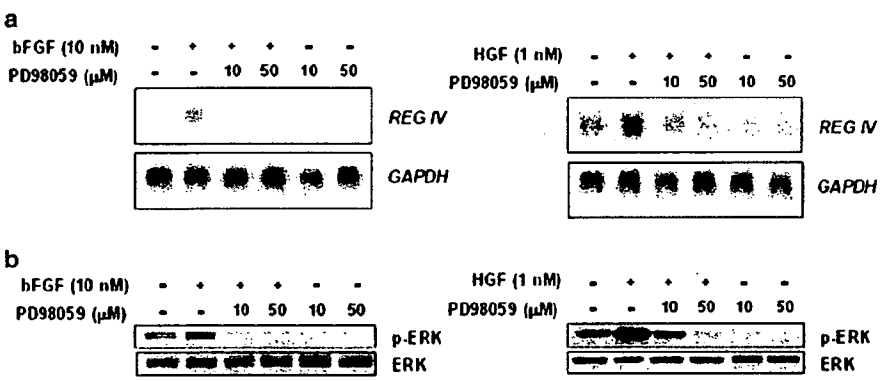


**Figure 5** Effects of bFGF and HGF on *REG IV* gene expression via the MAPK pathway in SW403 cells. (**a**) The cells were preincubated with 10 or 50 $\mu$M PD98059 for 1 h and stimulated with 10 nM bFGF or 1 nM HGF for 12 h. Total RNA (20 $\mu$g) was extracted and analyzed by Northern blotting using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

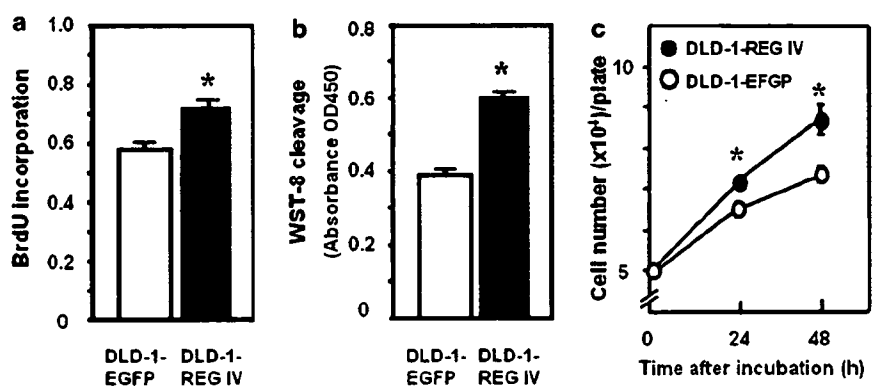


**Figure 6** Effects of *REG IV* gene induction on BrdU incorporation by (**a**) and growth of (**b**, **c**) human colon cancer cells. DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) or pIRES2-EGFP (DLD-1-EGFP; control) plasmids were used for both BrdU incorporation and cell growth assays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

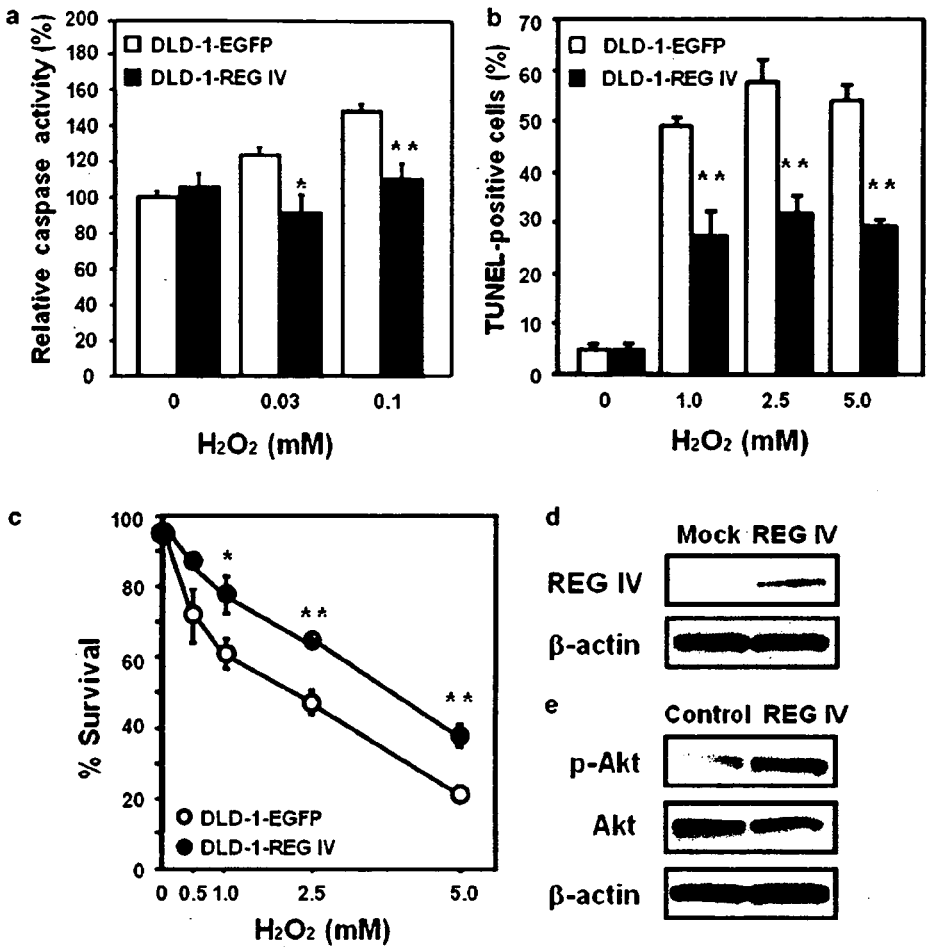


**Figure 7** Effects of *REG IV* gene induction on $H_2O_2$-induced apoptosis and cell death of human colon cancer cells. DLD-1 cells transfected with pIRES2-hREG IV (DLD-1-REG IV) or pIRES2-EGFP (DLD-1-EGFP; control) plasmids were treated with different concentration of H2O2 for 2 h and assessed for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

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

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

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

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

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

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

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