Effect of REG Iα protein on angiogenesis in gastric cancer tissues

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Abstract. Regenerating gene (REG) Iα is not only over-expressed in a subset of gastric cancers, but also involved in tumor progression. However, the mechanism by which (REG) Iα promotes tumor growth is not fully understood. In the present study, we investigated whether REG Iα plays a role in angiogenesis during the progression of gastric cancers. Expression of REG Iα and its receptor (EXTL3; exostoses like-3) was examined using immunohistochemistry in specimens of human gastric cancer. Microvessel density (MVD) in gastric cancer tissues was evaluated using an image analysis system after CD34 immunostaining. Relationships among clinicopathological features, REG Iα expression and MVD in gastric cancer tissues were analyzed. Effects of REG Iα protein on HUVEC cells in terms of proliferation and anti-apoptosis were assessed by WST-1 assay and FACS, respectively. Furthermore, the intracellular signaling by which REG Iα exerts its biological roles was examined *in vitro*. REG Iα expression was significantly related to lymph node metastasis and its receptor EXTL3 was ubiquitously expressed in not only the tumor cells, but also the tumor vessel cells in the gastric cancer tissues. MVD was significantly higher in gastric cancers that were REG Iα-positive than in those that were negative. Treatment with REG Iα protein promoted growth and anti-apoptosis through activation of the ERK and Akt signaling pathways in HUVEC cells, whereas these effects were attenuated by treatment with anti-REG Iα antibody. REG Iα protein may play a role in angiogenesis during progression of gastric cancer.

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Abbreviations: REG, regenerating gene; EXTL3, exostoses like-3; MVD, microvessel density; HUVEC, human umbilical vein endothelial cell; ERK, extracellular signal-regulated protein kinase; PI, propidium iodide; FITC, fluorescein isothiocyanate; FACS, fluorescence activated cell sorting; CD, cluster of differentiation

Key words: REG protein, gastric cancer, angiogenesis, EXTL3, microvessel

Introduction

The regenerating gene (Reg) was originally isolated from a complementary DNA library of rat regenerating pancreatic islets (1). Thereafter, its human homologue REG Iα was suggested to be involved in the pathophysiology of not only the gastrointestinal inflammation but also its associated cancer (2-4). Moreover, we previously clarified that REG Iα protein acts as a trophic and/or anti-apoptotic factor in the development of gastric cancer (5). With regard to the clinical significance of REG Iα expression, it has been reported that REG Iα is a useful marker for predicting the response to chemotherapy or prognosis in patients with gastric cancer (6-8).

Gastric cancer has a poor prognosis because of its marked propensity for invasion and metastasis. Gastric cancer tissues are composed of not only cancer cells but also stromal cells, and their interaction is thought to be crucial for tumor progression. Regarding the role of REG Iα protein, accumulating evidence suggests that the REG Iα receptor (EXTL3; exostoses like-3) is ubiquitously expressed in gastric cancer cells (9) and that REG Iα protein secreted from the cells promotes tumor cell growth or survival through an autocrine or paracrine mechanism (5). However, the effect of REG Iα protein on stromal cells remains unclear. Endothelial cells, which are an important stromal component in the tumor microenvironment, play a role in angiogenesis by interacting with the tumor cells, resulting in tumor progression (10). Therefore, in the present study, we investigated whether REG Iα protein promotes the growth and survival of the endothelial cells and examined the intracellular signaling by which REG Iα protein affects endothelial cell growth and survival. Moreover, to clarify the significance of REG Iα protein in angiogenesis, we investigated the expression of REG Iα and microvessel density (MVD) in gastric cancer tissues.

Materials and methods

Reagents and cell culture. Anti-Akt, anti-phospho-specific Akt (p-Akt; Ser473), anti-ERK, and anti-phospho-specific ERK (p-ERK) antibodies were purchased from Cell Signaling

Technology (Beverly, MA, USA). Anti-β-actin antibody was purchased from Sigma.

The human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD, USA) and maintained in EGM-2 medium containing the bullet kit according to the supplier's instructions. All the cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2.

Recombinant REG Iα protein was generated in insect cells using the Bac-to-Bac expression system (Invitrogen, Carlsbad, CA, USA) by Kitayama Labes (Ina, Japan). Full length human REG Iα cDNA was cloned and inserted into the pFastBac vector (Invitrogen). The constructed vector was then transformed into *E. coli* DH 10Bac, and recombinant Bacmid-REG Iα was produced by transposition. Then, *Spodoptera frugiperda* (*Sf9*) insect cells were infected with Bacmid-REG Iα to generate the recombinant baculoviruses carrying human REG Iα cDNA. The recombinant baculovirus particles were harvested in the culture supernatant, and used to infect *Sf9* insect cells in a large volume (1 L) of culture medium. The supernatant (crude extract) including the secreted REG Iα protein was then incubated with Ni-NTA agarose (Qiagen) and purified by elution through SP-Sepharose (GE Healthcare Life Science).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from each cell line using Trizol reagent (Invitrogen). Five micrograms of total RNA were reverse-transcribed using oligo dT primer (Applied Biosystems, Branchburg, NJ, USA) and 200 U of Superscript™ II reverse transcriptase (Invitrogen) in a total volume of 20 μl. For the following PCR, pairs of oligonucleotide primers for human EXTL3 (11) and human *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (9) were prepared. Human EXTL3: 5'-CAACCGATTCTTACCCTGG-3' (sense) and 5'-GGAAGTTCATGGCAATGTCC-3' (antisense); human GAPDH: 5'-GGCTGCTTTTAACTCTGGTA-3' (sense and 5'-ATGCCAGTGAGCTTCCCGT-3' (antisense). One microliter of RT product (cDNA) was amplified by PCR as previously described (11).

Western blot analysis. Following treatment with or without a reagent, the cells were lysed in protein extraction buffer as previously reported (12). Protein extract (25 μg) was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with a primary antibody and then with a peroxidase-conjugated secondary antibody. Proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

Tissue specimens and histological examination. A total of 31 gastric cancer tissues was obtained from specimens that were resected surgically at Dokkyo University School of Medicine. The tissue specimens were fixed in a 10% formalin solution and embedded in paraffin. This study was approved by the Dokkyo University Surgical Pathology Committee and an informed consent was obtained from all the participant patients.

Multiple hematoxylin and eosin-stained sections of all 31 lesions were examined (Table I). The following factors were determined for all the patients and lesions; age, sex, tumor size, tumor location, Lauren's histological classification, tumor invasion, lymph node metastases and tumor stage according to the system of the American Joint Committee on Cancer.

Table I. Clinicopathological features of the patients with gastric cancer.

Gender		
	Man	22 (71.0%)
	Woman	9 (29.0%)
Age (yr, mean ± SE)		65.8±1.7
Tumor location		
	Lower	7 (22.6%)
	Mid	12 (38.7%)
	Upper	12 (38.7%)
Lauren's classification		
	Intestinal type	9 (29.0%)
	Diffuse type	22 (71.0%)
Stage		
	I	5 (16.1%)
	II	3 (9.7%)
	III	12 (38.7%)
	IV	11 (35.5%)
Lymphatic invasion		
	None	1 (3.2%)
	Present	30 (96.8%)
Venous invasion		
	None	4 (12.9%)
	Present	27 (87.1%)
Lymph node metastasis		
	None	5 (16.1%)
	Present	26 (83.9%)

Immunohistochemistry. Immunohistochemical staining for CD34 and REG Iα was performed with an Envision kit (DAKO, Kyoto, Japan) as described previously (3,13), using anti-CD34 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, Japan) and anti-REG Iα antibody (1:500). Finally, the sections were incubated in 3,3'-diaminobenzide tetrahydrochloride with 0.05% H_2O_2 for 3 min and then counterstained with Mayer's hematoxylin. To evaluate the immunoreactivity of REG Iα protein, at least 500 tumor cells were counted in five different visual fields for each sample of the cancerous tissues. A specimen was considered positive for REG Iα protein if 20% of the tumor cells were positively stained (9). To evaluate angiogenesis in the tumors, MVD was assessed by immunostaining with the anti-CD34 antibody as described above. Five different fields (x200) were digitally photographed with a high-resolution microscope (DP20, Olympus, Tokyo, Japan), and the obtained images were analyzed using NIH ImageJ1.47 image analysis software (http://rsbweb.nih.gov/ij). MVD was quantified as the percentage of the microvascular area relative to the tumor stroma in each image and the results were averaged (14).

Cell growth and apoptosis assay. HUVECs were seeded in complete medium in 96-well plates (1×10^4 cells/well) and 6-well

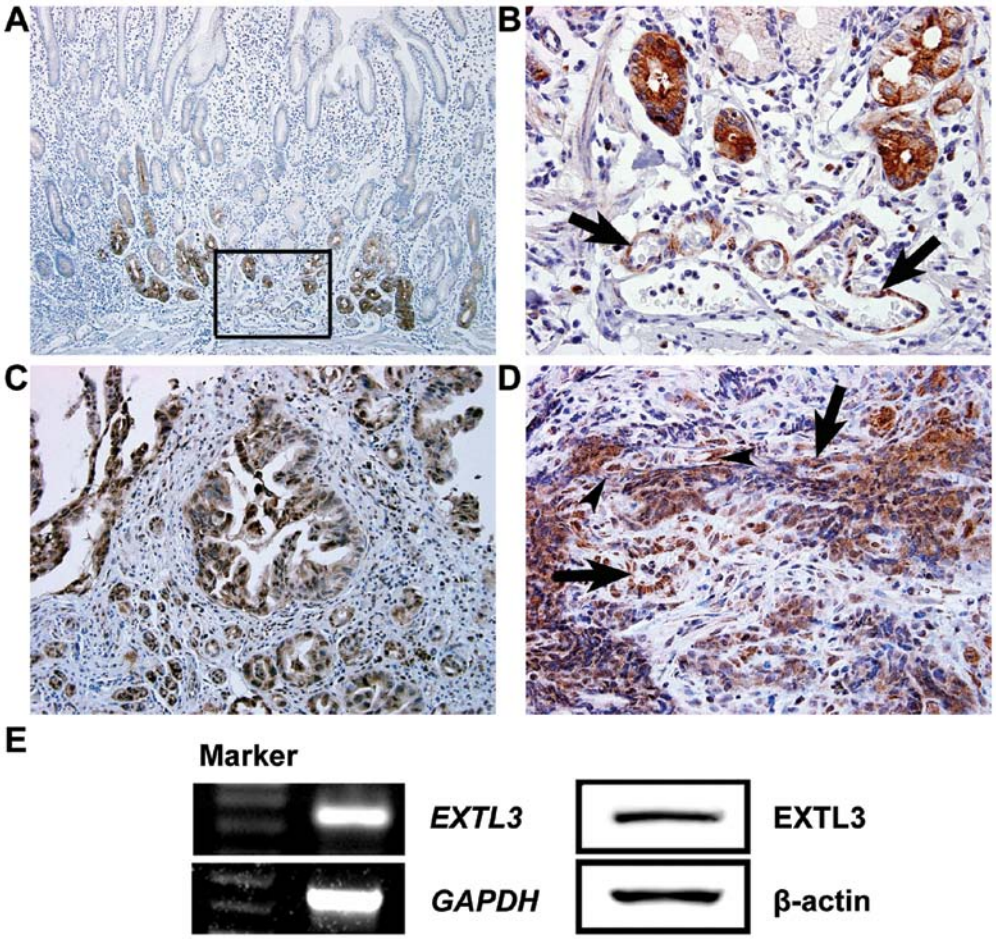

Figure 1. Expression of EXTL3 (REG Iα receptor) in the epithelium (A) and endothelial cells (B) in normal gastric mucosa. (A) EXTL3 is expressed strongly in the basal and weakly in the middle part in the gastric epithelium. (B) Magnified view of the square in (A). Arrows indicate the expression of EXTL3 in the endothelial cells. (C and D) Expression of EXTL3 in gastric cancer tissues. (C) Well-differentiated adenocarcinoma. (D) Poorly-differentiated adenocarcinoma. EXTL3 is expressed in tumor vessel cells (arrowheads) and cancer cells showing capillary formation (arrows). (E) Detection of EXTL3 mRNA and its gene product in HUVEC cells by RT-PCR (left) and western blot analysis (right), respectively.

plates ($2x10^5$ cells/well) for cell growth and apoptosis assay, respectively. After 24 h, the cells were washed in a serum-free medium and then incubated with or without REG Iα protein for the indicated time. For the cell growth assay, the treated cells were incubated with Premix WST-1 reagent (Takara, Tokyo, Japan) for 1 h and the plates were read at 450 and 600 nm in a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For the apoptosis assay, the treated cells were collected, washed with PBS, and incubated with Annexin V-FITC and propidium iodide (PI) in binding buffer in accordance with the manufacturer's protocol (MEBCYTO-Apoptosis Kit; MBL, Ina, Japan). The stained cells were analyzed on a FACScalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and the data obtained were analyzed using CellQuest software (Becton-Dickinson).

Statistical analysis. All values were expressed as the mean ± SEM. The data for MVD were analyzed using unpaired two-tailed *t*-test. Chi-squared analyses were performed to determine the correlation between various pathological parameters and Fisher's exact test was also performed when necessary. P-values of <0.05 were considered to indicate statistical significance.

Results

Expression of EXTL3 and its gene product in the endothelial cells in normal gastric tissues and gastric cancer. EXTL3 was ubiquitously expressed not only in the epithelial cells, but also in the endothelial cells in the normal gastric mucosa (Fig. 1A and B). In gastric cancer tissues, EXTL3 was expressed in tumor vascular cells as well as cancer cells (Fig. 1C and D).

Before examining the effect of REG Iα protein on the endothelial cells, we tested the expression of EXTL3 in HUVEC. Subsequently, we confirmed that expression of *EXTL3* and its gene product was detectable in the cells by RT-PCR and western blot analysis (Fig. 1E), suggesting that HUVECs have the capability of responding to REG Iα stimulation.

REG Iα protein activates the phosphorylation of ERK and Akt in HUVEC cells. The effect of REG Iα protein on intracellular signaling was investigated in the HUVEC cells. The expression of p-ERK and p-Akt was enhanced by REG Iα stimulation (10-100 nM) (Fig. 2A). The expression of p-ERK peaked in the HUVEC cells at 15 min after REG Iα stimulation, and that of p-Akt was enhanced from 15 min and sustained for

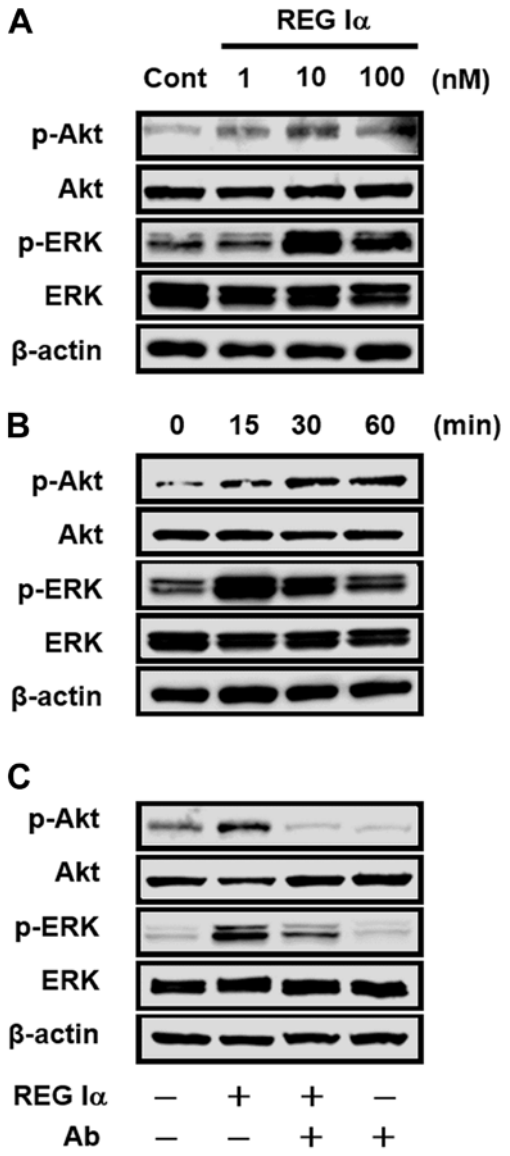

Figure 2. Effect of REG Iα treatment on intracellular signaling in endothelial cells. (A) Dose-dependent effect of REG Iα on phosphorylation of Akt and ERK in HUVEC cells. HUVEC cells ($2.5x10^5$) were cultured in 35-mm dishes and treated with various concentrations of REG Iα protein for 30 min. Extracted protein was analyzed by western blot analysis, as described in Materials and methods. (B) Time course changes in Akt and ERK phosphorylation in HUVEC cells treated with REG Iα. HUVEC cells were similarly treated with REG Iα protein (10 nM) for the indicated times. (C) Effect of anti-REG Iα antibody on REG Iα protein-induced Akt and ERK phosphorylation in HUVEC cells. HUVEC cells were pretreated with anti-REG Iα antibody (Ab; 50 μg/ml) for 45 min and then stimulated with REG Iα protein (10 nM) for 30 min.

60 min (Fig. 2B). We then examined whether anti-REG Iα antibody inhibits the REG Iα-induced signaling in HUVEC cells. As shown in Fig. 2C, the basal level of p-ERK and p-Akt expression was decreased by treatment with REG Iα antibody. Moreover, the increased expression of p-ERK and p-Akt in REG Iα-treated HUVEC cells was attenuated by concomitant administration of anti-REG Iα antibody.

REG Iα protein promotes HUVEC cell growth and anti-apoptosis. To clarify the role of REG Iα protein in angiogenesis, we examined the growth and anti-apoptosis effects of REG Iα protein on HUVEC cells *in vitro*. The rate of WST-1 cleavage was significantly and dose-dependently increased in REG Iα-treated HUVEC cells (Fig. 3A). Conversely, the increase in the level of WST-1 cleavage in REG Iα-treated cells was significantly reduced to almost the control level by addition of anti-REG Iα antibody (Fig. 3B).

In control preparations, deprivation of growth factors in complete culture medium induced cell apoptosis and death. However, HUVEC cells treated with REG Iα protein (10 nM) showed significantly lower Annexin V positivity, than the control cells (Fig. 3C and D). Similarly, the percentage of PI-positive cells was significantly lower in the REG Iα-treated preparations than in the controls (Fig. 3C and D). On the other hand, the decrease of Annexin V or PI positivity in REG Iα-treated HUVEC cells was restored to the control level by concomitant administration of anti-REG Iα antibody (Fig. 3D).

Relationship between REG Iα expression and MVD in gastric cancer tissues. Among 31 samples of gastric cancer tissues, 19 (61.3%) were positive for REG Iα expression. Expression of REG Iα was significantly associated with the prevalence of lymph node metastasis and tended to correlate with the tumor stage (Table II). MVD was significantly higher in gastric cancers at an advanced stage. In addition, MVD tended to be higher in gastric cancers with lymph node metastasis. Furthermore, we investigated the relationship between REG Iα expression and MVD and observed that MVD was significantly higher in REG Iα-positive gastric cancers (Fig. 4).

Discussion

It has been reported that REG Iα is overexpressed in various malignancies including cancers of the stomach (3,6,9), colorectum (15), bile duct (16) and pancreas (17). Furthermore, microarray analyses have revealed that REG Iα expression is markedly enhanced in gastric cancer tissues (18) and in fact we have previously shown that REG Iα protein acts on gastric cancer cells as a growth and/or anti-apoptotic factor (5). Although the receptor for REG Iα protein, which is identical to EXTL3, has been discovered fairly recently (19), its pathophysiological roles are poorly understood. In the present study using immunohistochemistry, we have demonstrated that EXTL3 is expressed in gastric cancer cells, in accordance with a previous study indicating that *EXTL3* is ubiquitously expressed in gastric cancer cells *in vitro* (9), which would account for the observed effects of REG Iα protein on gastric cancer cells. Interestingly, our immunohistochemical analysis also revealed that EXTL3 was expressed in tumor vessel cells and we confirmed the expression of EXTL3 in HUVEC cells *in vitro*. Thus, the present study indicates for the first time that REG Iα protein may act on not only gastric cancer cells but also tumor vessel cells, which are an important component associated with tumor progression.

In a series of *in vitro* studies, we have investigated the possible role of REG Iα on the human endothelial cells and have shown that REG Iα protein promotes the proliferation of the endothelial cells. Furthermore, in the present study, we clarified that REG Iα protein has an anti-apoptotic effect on the endothelial cells. Thus, REG Iα protein appears to act on

Table II. Relationship between clinicopathological features and REG Iα expression or MVD in patients with gastric cancer.

	Number of REG Iα-positive/ total number of patients	P-value	MVD	P-value
Tumor location		0.0575		NS
Lower	7/7 (100%)		9.44±2.39	
Mid	6/12 (50.0%)		8.87±2.03	
Upper	6/12 (50.0%)		12.08±2.26	
Lauren's classification		0.2181		0.5414
Intestinal type	4/9 (44.4%)		8.99±2.46	
Diffuse type	15/22 (68.2%)		10.75±1.52	
Stage		0.1088		0.0459
I/II	3/8 (37.5%)		5.94±1.39	
III/IV	16/23 (69.6%)		11.74±1.55	
Lymphatic invasion		0.2009		NS
None	0/1 (0.0%)		9.78	
Present	19/30 (63.3%)		10.26±1.33	
Venous invasion		0.6194		0.730
None	2/4 (50.0%)		9.06±2.56	
Present	17/27 (63.0%)		10.42±1.43	
Lymph node metastasis		0.0385		0.0614
None	1/5 (20.0%)		4.80±1.32	
Present	18/26 (69.2%)		11.29±1.42	

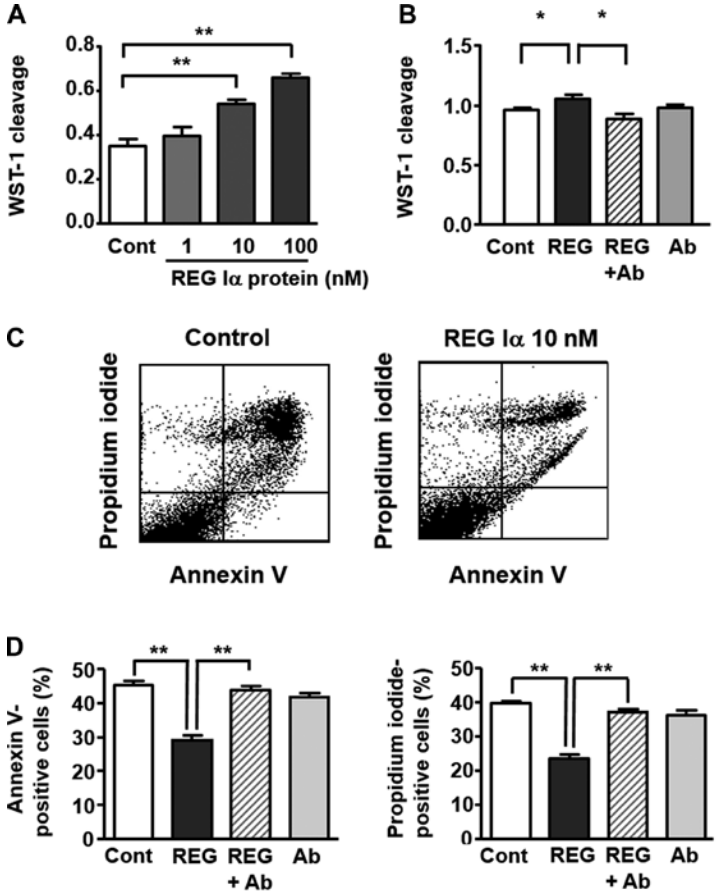

Figure 3. Effect of REG Iα on growth (A and B) and apoptosis (C and D) of the endothelial cells. (A) The dose-dependent effect of REG Iα on growth of the HUVEC cells. (B) Effect of anti-REG Iα antibody (50 μg/ml) on HUVEC cell growth promoted by REG Iα protein (10 nM). (C) Representative graphs of FACS analysis using Annexin V-FITC and propidium iodide staining. HUVEC cells were treated with REG Iα protein (10 nM) and were evaluated as described in Materials and methods. (D) The effect of anti-REG Iα antibody (50 μg/ml) on REG Iα (10 nM)-induced anti-apoptosis and survival of HUVEC cells. All the results are presented as the mean ± SEM of four independent experiments. Significant differences between two groups at *P<0.05 and **P<0.01.

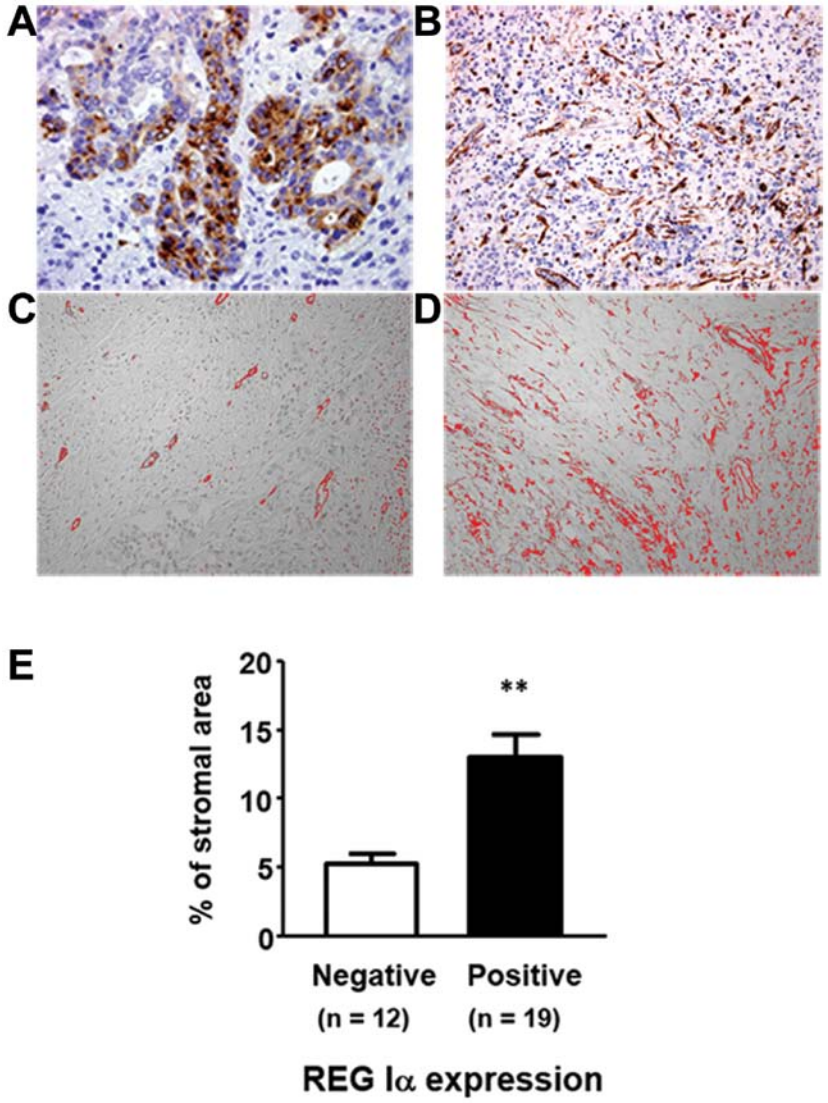

Figure 4. Evaluation of REG Iα expression and MVD in gastric cancer. (A and B) Immunostaining of REG Iα and CD34 in gastric cancer tissue. (A) REG Iα and (B) CD34 are expressed in tumor cells and tumor vessel cells, respectively. (C and D) Representative images showing microvascular areas (CD34; red) in (C) REG Iα-negative and (D) -positive gastric cancer. (E) Relationship between REG Iα expression and MVD in gastric cancer tissues. **Significantly greater than in the REG Iα-negative preparation ($P<0.01$). MVD, microvessel density.

not only the gastric cancer cells (5), but also the endothelial cells as a growth and/or anti-apoptotic factor. In addition, to clarify how REG Iα protein exerts its effects on endothelial cells, we examined the signaling pathways activated by REG Iα protein in HUVEC cells. As shown in Fig. 2, REG Iα stimulation enhanced the phosphorylation of ERK and Akt in HUVEC cells, similarly to stimulatory effect of REG Iα protein on gastric cancer cells (5,20). Conversely, treatment with anti-REG Iα antibody attenuated the enhancement of ERK and Akt phosphorylation and simultaneously suppressed the growth-promoting and anti-apoptotic effects of REG Iα on HUVEC cells. These findings suggest that REG Iα protein acts on endothelial cells as a growth and/or anti-apoptotic factor via the ERK and Akt signaling pathways.

Angiogenesis is an important process associated with tumor progression. In this context, REG Iα protein may promote tumor progression through its growth-promoting and/or anti-apoptotic effect on the endothelial cells. To address this issue, we investigated the expression of REG Iα and microvessel density in gastric cancer tissues. Clinicopathological analyses revealed that expression of REG Iα was significantly associated with the prevalence of lymph node metastasis. Moreover, gastric cancers that were REG Iα-positive showed a significantly higher MVD than those that were negative. Although confirmation of these clinicopathological data may be necessary in a larger study, the present findings suggest that REG Iα protein is indeed involved in gastric cancer angiogenesis. In addition, since receptors for REG Iα protein are ubiquitously expressed not only in gastric cancer, but also in its endothelial cells, REG Iα protein may contribute at least in part to tumor progression in REG Iα-positive gastric cancer.

In summary, we have shown that receptors for REG Iα are expressed not only in tumor cells, but also tumor vessel cells in gastric cancer, and that angiogenesis is significantly promoted in gastric cancers that are REG Iα-positive. Moreover, we have clarified that REG Iα protein exerts growth-promoting and anti-apoptotic effects on endothelial cells via ERK and Akt signaling. These findings suggest that REG Iα protein plays an

important role in angiogenesis during progression of gastric cancer.

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