Expression and roles of keratinocyte growth factor and its receptor in esophageal cancer cells

MASANORI YOSHINO[1,2], TOSHIYUKI ISHIWATA[1], MASANORI WATANABE[2], TETSURO MATSUNOBU[2], OSAMU KOMINE[2], YURI ONO[1], TETSUSHI YAMAMOTO[1], TAKENORI FUJII[1], KOSHI MATSUMOTO[3], AKIRA TOKUNAGA[2] and ZENYA NAITO[1]

[1]Department of Pathology, Integrative Oncological Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602; [2]Institute of Gastroenterology, and [3]Division of Pathology, Musashikosugi Hospital of Nippon Medical School, 1-396 Kosugicho, Nakahara-ku, Kawasaki, Kanagawa 211-8533, Japan

Received May 11, 2007; Accepted July 6, 2007

Abstract. The keratinocyte growth factor receptor (KGFR), also known as FGFR2 IIIb, is mainly localized in epithelial cells and is activated by the keratinocyte growth factor (KGF) that is predominantly synthesized by mesenchymal cells. In this study, we examined the roles of KGFR and KGF in human esophageal cancer (EC). In noncancerous esophageal tissues, KGFR was localized in epithelial cells from the basal region of the epithelium to the lower one-third of the epithelium, and KGF was weakly localized in the basal to parabasal epithelial cells. On the other hand, Ki-67 was localized in the parabasal cells. In EC tissues, KGFR and KGF were expressed in cancer cells in 22 and 37 of 54 patients, respectively. The coexpression of KGFR and KGF in cancer cells was detected in 14 of 54 (26%) patients. Clinicopathologically, KGFR expression correlated with the well-differentiated cell type of EC ($p<0.001$), and KGF expression correlated with lymphatic invasion and lymph node metastasis ($p=0.004$ and 0.021, respectively). The coexpression of KGFR and KGF in cancer cells correlated with the well-differentiated cell type of EC ($p=0.001$). KGFR-positive, KGF-positive and KGFR/KGF coexpression patients tended to have shorter survival rates, but the survival rates were not statistically significantly different ($p=0.44$, 0.059 and 0.112, respectively). In human EC cell lines (TE-1, TE-8 and TE-11), KGFR mRNA was expressed but no KGF mRNA was detected. The KGFR mRNA level was highest in TE-1 cells, derived from well-differentiated SCC and lowest in TE-8 cells. KGFR was detected in the cancer cell lines by Western blot analysis. Recombinant human KGF significantly stimulated the growth of TE-8 and -11 cells, derived from moderately differentiated SCC, but had no effect on TE-1 cell growth. These results suggest that KGFR expression correlates with the differentiation of a normal esophageal epithelium and the well-differentiated cell type of EC. On the other hand, KGF may induce the growth of some EC cells in a paracrine manner and closely correlates with lymphatic invasion and lymph node metastasis.

Correspondence to: Dr Zenya Naito, Department of Pathology, Integrative Oncological Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan
E-mail: naito@nms.ac.jp

Key words: keratinocyte growth factor/fibroblast growth factor-7, keratinocyte growth factor receptor/fibroblast growth factor receptor 2 IIIb, esophageal cancer, immunohistochemistry

Introduction

The fibroblast growth factor (FGF) family has at least 22 members, which range in molecular mass from 17 to 34 kD and share 13-71% amino acid identities (1,2). FGFs have been implicated in the regulation of cell differentiation, proliferation, migration and survival in many different cell types. The biological activities of FGFs are mediated by FGF receptors (FGFRs) that undergo dimerization and autophosphorylation after ligand binding. FGFRs are encoded by four distinct genes (designated *Fgfr1, 2, 3* and *4*) characterized by two or three extracellular immunoglobulin (Ig)-like loops, a single transmembrane region and a cytoplasmic tyrosine kinase domain. The third Ig-like loop is encoded by two or three exons of *Fgfr1, 2* and *3*, enabling the synthesis of alternatively spliced isoforms (3,4). The two different receptor isoforms of *Fgfr2* are designated FGFR2 IIIb, also known as the keratinocyte growth factor receptor (KGFR), and FGFR2 IIIc. KGFR is specifically localized in epithelial cells whose growth is induced by FGF-1, FGF-3, the keratinocyte growth factor (KGF/FGF-7) or FGF-10 (KGF-2), whereas FGFR2 IIIc binds FGF-1, -2, -4, -6 and -9 and is expressed mainly in mesenchymal cells (4,5).

Mice null for the *Fgfr2 IIIb* isoform but still retaining *Fgfr2 IIIc* exhibit dysgenesis of the kidneys, salivary glands, adrenal glands, thymus, pancreas, skin, otic vesicles, glandular stomach and hair follicles, and agenesis of the lungs, anterior pituitary gland, thyroid gland, teeth and limbs (6-8). KGFR is expressed in epithelial cells throughout the gastrointestinal (GI) tract, and an intraperitoneal injection of KGF enhances normal intestinal epithelial cell growth (9,10). In the early stage of esophageal ulcer healing, the expression levels of

KGF mRNA synthesized by stromal cells and KGFR mRNA in epithelial cells markedly increase, and a single injection of recombinant human KGF (rhKGF) further enhances cell proliferation and accelerates ulcer healing (11). Regarding cancerous tissues, KGFR expression has been reported in pancreatic and colorectal cancer cells (12-14). Pancreatic cancer cell line growth is stimulated by rhKGF administration (15). Furthermore, a dose-dependent cell growth stimulated by KGF has been observed in well-differentiated colorectal cancer cell lines (16). KGF enhances the proliferation of gastric carcinoma cells derived from scirrhous tumors (17). In esophageal cancer (EC), 13 of the EC cell lines express KGFR mRNA (18). These lines of evidence suggest that the KGF-KGFR pathway plays an important role in the growth of epithelial cells in the GI tract, as well as cancer cells.

On the other hand, the growth of most human cancer cell lines (30/35) is not significantly affected by rhKGF (19). KGF fails to effectively stimulate the growth of two of three pancreatic cancer cell lines and of colorectal cancer cell lines, established from undifferentiated cancer or from a metastatic nodule (13,15). KGF-transfected prostate epithelial cells are not tumorigenic in nude mice (20). A stable transfectant of *Kgfr* of salivary adenocarcinoma cells induces cancer cell differentiation and apoptosis (21).

Recently, the U.S. Food and Drug Administration (FDA) has approved the truncated form of recombinant KGF-palifermin for clinical use for patients with hematologic malignancies. The use of palifermin in patients with hematologic malignancies reduces the incidence and duration of severe oral mucositis after intensive chemoradiotherapy (22). The safety and efficacy of palifermin in nonhematologic malignancies including EC have not yet been established; hematologic malignancies do not have KGFR, but most cancer cells do (23). In a randomized phase II clinical trial in patients with advanced colorectal cancer who were treated with 5-FU and leucovorin, patients who received palifermin had a lower incidence of ulcerative oral mucositis than those who did not receive palifermin (24).

In this study, we examined the expression and roles of KGFR and KGF in EC cells. We report that KGFR expression and the coexpression of KGFR and KGF in cancer cells correlate with the differentiated type of EC, and that KGF expression in cancer cells correlates with lymphatic invasion and lymph node metastasis. The KGFR-positive, KGF-positive and KGFR/KGF coexpression patients tend to have shorter survival rates than KGFR-, KGF- and KGFR/KGF-negative patients, respectively. Furthermore, exogenous KGF induces the growth of some EC cells in a paracrine manner.

Materials and methods

Materials. The following were purchased: goat anti-rabbit IgG-HRP secondary antibody from American Qualex (San Clemente, CA, USA); transcriptor first-strand cDNA synthesis kit and LightCycler-FastStartDNA Master SYBR-Green I mix from Roche Diagnostics GmbH (Mannheim, Germany); antibiotic-antimycotic and Superscript III reverse transcriptase from Invitrogen Corp. (Carlsbad, CA, USA); an Immobilon PVDF membrane and Immobilon Western chemiluminescent HRP substrates from Millipore (Yonezawa, Japan); M-PER (mammalian protein extraction reagent) from Pierce (Rockford, IL, USA); Cell counting kit-8 from Wako Pure Chemicals (Osaka, Japan); Takara RNA polymerase chain reaction (PCR) kit (AMV) Ver. 3.0 from Takara (Tokyo, Japan); RNeasy mini kit from Qiagen (Tokyo, Japan); recombinant human KGF from Pepro Tech EC Ltd. (London, UK); Histofine Simple Stain Max PO (R), (G) and (M) kits from Nichirei (Tokyo, Japan); superfrosted slides with an MAS coat from Matsunami Glass Ind., Ltd. (Osaka, Japan); a malinol mounting medium from Muto Chemical Co., (Tokyo, Japan); mouse monoclonal anti-Ki-67 antibody from Dako Cytomation (Kyoto, Japan); and goat polyclonal anti-FGF-7 antibodies from R&D Systems Inc. (Westerville, OH, USA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

Human EC tissues. EC tissue samples were obtained from 6 female and 48 male patients during esophagectomy for advanced EC at the Institute of Gastroenterology, Musashikosugi Hospital of Nippon Medical School (Table I). The mean age of the cancer patients was 64.1 years (range, 38-86 years). By TNM classification, 2 patients had Stage 0 EC, 4 patients had Stage I, 12 patients had Stage II, 21 patients had Stage III and 15 patients had Stage IV. Histologically, 20 well-differentiated, 19 moderately differentiated and 15 poorly differentiated squamous cell carcinomas (SCCs) were observed. Noncancerous esophageal tissue samples were also obtained from the surgical margin at least 10 cm away from each cancerous growth from the same patient. The tissues were fixed in 15% formalin for 24-48 h and then embedded in paraffin. All studies were approved by the Human Ethics Committee of the Musashikosugi Hospital of Nippon Medical School.

Human EC cell lines. TE-1, TE-8 and TE-11 EC cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). TE-1 cells were established from well-differentiated SCC, and TE-8 and TE-11 cells were established from moderately differentiated SCC. The cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B at 37˚C in a humidified 5% CO_2 atmosphere.

Immunohistochemistry. The anti-KGFR antibody used was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids of human KGFR, as previously reported (25). Paraffin-embedded sections (3 μm thick) were subjected to immunostaining using a Histofine Simple Stain Max PO (R) or (G) kit. After deparaffinization, the endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min. The tissue sections were incubated with the appropriate antibody for 16 h at 4˚C (1:1000 dilution for the anti-KGFR antibody, 1:100 dilution for the anti-KGF antibody) using phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The bound antibodies were detected with the Simple Stain Max PO (G) or (R) reagent using diaminobenzidine tetrahydrochloride (DAB) as the substrate. The sections were counterstained

Table I. Correlation of clinicopathological features and KGFR, KGF or coexpression of KGFR and KGF in esophageal cancers.

Variables		KGFR			KGF			KGFR and KGF		
	No.	No.	%	P	No.	%	P	No.	%	P
Gender										
Female	6	3	50.0	NS	4	66.7	NS	2	33.3	NS
Male	48	19	39.6		33	68.8		12	25.0	
Age										
<65 years	25	12	48.0	NS	20	80.0	NS	9	36.0	NS
≥65 years	29	10	34.5		17	58.6		5	17.2	
Histological features										
Well-differentiated	20	16	80.0	<0.001	13	65.0	NS	11	55.0	<0.001
Moderately differentiated	19	4	21.1		16	84.2		2	10.5	
Poorly differentiated	15	2	13.3		8	53.3		1	6.7	
Macroscopic type										
0 (superficial)	7	4	57.1	NS	2	28.6	NS	0	0.0	NS
1 (protruding)	7	1	14.3		6	85.7		2	28.6	
2 (ulcerative and localized)	17	8	47.1		14	82.4		7	41.2	
3 (ulcerative and infiltrative)	20	9	45.0		12	60.0		5	25.0	
4 (diffusely infiltrative)	1	0	0.0		1	100.0		0	0.0	
5 (unclassifiable)	2	0	0.0		2	100.0		0	0.0	
Depth of invasion										
T1 (LPM, MM, SM)	8	3	37.5	NS	3	37.5	NS	0	0.0	NS
T2 (MP)	13	5	38.5		9	69.2		5	38.5	
T3 (Ad)	29	13	44.8		23	79.3		8	27.6	
T4 (Adj)	4	1	25.0		2	50.0		1	25.0	
Lymphatic invasion										
(-)	8	3	37.5	NS	2	25.0	0.004	1	12.5	NS
(+)	46	19	41.3		35	76.1		13	28.3	
Blood vessel invasion										
(-)	41	15	36.6	NS	29	70.7	NS	9	22.0	NS
(+)	13	7	53.8		8	61.5		5	38.5	
Lymph node metastasis										
(-)	17	6	35.3	NS	8	47.1	0.021	2	11.8	NS
(+)	37	16	43.2		29	78.4		12	32.4	
Location of the lesion										
Cervical esophagus	3	0	0.0	NS	3	100.0	NS	0	0.0	NS
Upper thoracic esophagus	6	2	33.3		5	83.3		2	33.3	
Middle thoracic esophagus	27	14	51.9		15	55.6		8	29.6	
Lower thoracic esophagus	17	6	35.3		13	76.5		4	23.5	
Abdominal esophagus	1	0	0.0		1	100.0		0	0.0	
Stage grouping										
0	2	0	0.0	NS	1	50.0	NS	0	0.0	NS
I	4	2	50.0		1	25.0		0	0.0	
II	12	3	25.0		8	66.7		2	16.7	
III	21	10	47.6		16	76.2		8	38.1	
IV	15	7	46.7		11	73.3		4	26.7	

NS, not significant.

with Mayer's hematoxylin. For Ki-67 immunostaining, the tissue sections were preheated in 10 mM citrate buffer (pH 6.0) for 15 min at 121˚C in an autoclave oven. Then, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min. The tissue sections were incubated with anti-Ki-67 (dilution, 1:100) antibody in PBS containing 1% BSA for 16 h at 4˚C. The bound antibodies were detected using the Simple Stain Max PO (M) reagent with DAB as the substrate. The sections were lightly counterstained with Mayer's hematoxylin. Positivity for KGFR and KGF was classified according to the percentage of positive tumor cells regardless of staining intensities: when KGFR staining was observed in the cytoplasm or membrane of >10% of the tumor cells, the patients were designated KGFR-positive (26,27); when KGF staining was observed in the cytoplasm of >30% of the tumor cells, the patients were designated KGF-positive (28). Two investigators (M.Y. and T.I.) separately evaluated all the specimens in a blind manner.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNeasy mini kit according to the protocol of the manufacturer. Then, cDNA synthesis and RT-PCR were performed using the Takara RNA PCR kit. The primer pair used for KGFR cDNA preparation was derived from sequences of human KGFR cDNA, corresponding to nucleotides (nts) 1580-1603 (5'-TCT-CAA-GCA-CTC-GGG-GAT-AAA-TAG-3') and 1839-1859 (5'-CGT-GTT-CTT-CAT-TCG-GCA-CAG-3') (280 bp, accession no. NM_022970). The primers used for KGF corresponded to nts 411-430 (5'-ATG-TTA-TTC-ATG-AAC-ACC- CG-3') and 1046-1065 (5'-CTG-CTG-GAA-CTG-GGT-TCT-TT-3') of the human KGF cDNA (655 bp, NM_002009). ß-actin mRNA, as the positive control, was amplified using the following primer pairs: nts 331-353 (5'-GCA-CCA-CAC-CTT-CTA-CAA-TGA-GC-3') and 472-493 (5'-TAG-CAC-AGC-CTG-GAT-AGC-AAC-G-3') (163 bp, accession no. NM_001101). PCR was carried out in a Takara PCR thermal cycler MP (Takara) for 2 min at 94˚C and then for 35 cycles, each consisting of 30 sec at 94˚C, 30 sec at 60˚C and 1 min at 72˚C. Before cDNA synthesis, total RNA was run on a denatured gel to eliminate the possibility of RNA degradation during the extraction steps, and then used for transcription. As a negative control, RNA without RT was used as the template for PCR.

Quantitative RT-PCR. One microgram of total RNA sample was used for RT with the transcriptor first-strand cDNA synthesis kit following the manufacturer's protocol. Quantitative RT-PCR (qRT-PCR) was performed using a LightCycler-FastStartDNA Master SYBR-Green I system. The primer pairs used for KGFR and ß-actin were the same as those employed in RT-PCR. Twenty microliters of the PCR mixture containing 2 μl of template cDNA, 3 mM $MgCl_2$, 0.5 μM of each primer and LightCycler-FastStartDNA Master SYBR-Green I mix was loaded to the capillary tube. qRT-PCR was carried out in a LightCycler, and the PCR products were analyzed using LightCycler Data Analysis software Ver. 3.5 (Roche Diagnostics GmbH). To confirm amplification specificity, the PCR products were used for melting curve analysis. Results were expressed as KGFR/ß-actin, an internal standard concentration ratio. The optimized program involved denaturation at 95˚C for 10 min, followed by 45 cycles of amplification (at 95˚C for 10 sec, 58˚C for 10 sec, and 72˚C for 12 sec) for KGFR, and by 40 cycles of amplification (at 95˚C for 10 sec, 64˚C for 10 sec, and 72˚C for 7 sec) for ß-actin.

Western blot analysis. The same anti-KGFR antibody used for immunohistochemical analysis was utilized for Western blot analysis. Protein extraction was performed using the M-PER (mammalian protein extraction reagent), according to the manufacturer's protocol. Proteins from the EC cell lines were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under the reducing condition, and the separated proteins were transferred onto Immobilon PVDF transfer membranes. The membranes were preincubated with 5% skim milk/TBS-T for 2 h and then incubated for 16 h with the anti-KGFR antibody (dilution, 1:2000). The membranes were sequentially washed and incubated with the secondary HRP-conjugated anti-rabbit IgG antibody for 60 min. After washing, the blots were visualized by enhanced chemiluminescence and detected using the ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). To confirm almost equal loads of the proteins, the membrane was washed and reprobed with the anti-ß-actin antibody.

Effects of rhKGF on EC cell growth. TE-1, TE-8 and TE-11 EC cells were plated on a 96-well plate at a density of $2x10^3$ cells/well and cultured for 24 h in RPMI-1640 medium containing 10% FBS. These cells were then cultured in the same medium, with 0, 1, 10 and 100 ng/ml rhKGF for 48 h. The growth rates of the cells were evaluated using cell counting kit-8.

Statistical analysis. To analyze the correlation between KGFR expression and clinicopathological features, the χ^2 test or Mann-Whitney U test was used. Group comparisons were analyzed by one-way analysis of variance (ANOVA). The Tukey-Kramer test was used to compare the expression level of KGFR mRNA in EC cell lines and the Dunnett test was used to assess the effect of rhKGF on EC cell growth (Statview version 4.5). The mean ± standard error (SE) of two separate experiments was evaluated. $P<0.05$ was considered statistically significant.

Results

Immunohistochemical analysis of KGFR and KGF in EC. To confirm the localization of KGFR in human EC tissues, immunohistochemical staining was performed. In noncancerous esophageal tissues, KGFR was localized in the basal region to the lower one-third of squamous epithelial cells (Fig. 1A). KGF was weakly localized in the basal region to the parabasal region of the squamous epithelium (Fig. 1B). Ki-67 was localized in the parabasal region of the squamous epithelium (Fig. 1C). In EC tissues, KGFR was expressed in the cell membrane or cytoplasm of cancer cells adjacent to keratinizing lesions or large squamous pearls (Fig. 2A). KGF was expressed in the cytoplasm of cancer cells (Fig. 2B). KGFR and KGF expression in 22 and 37 of the 54 (41 and 69%, respectively) patients was detected in cancer cells, and the coexpression of KGFR and KGF in the cancer cells was detected in 14 of the

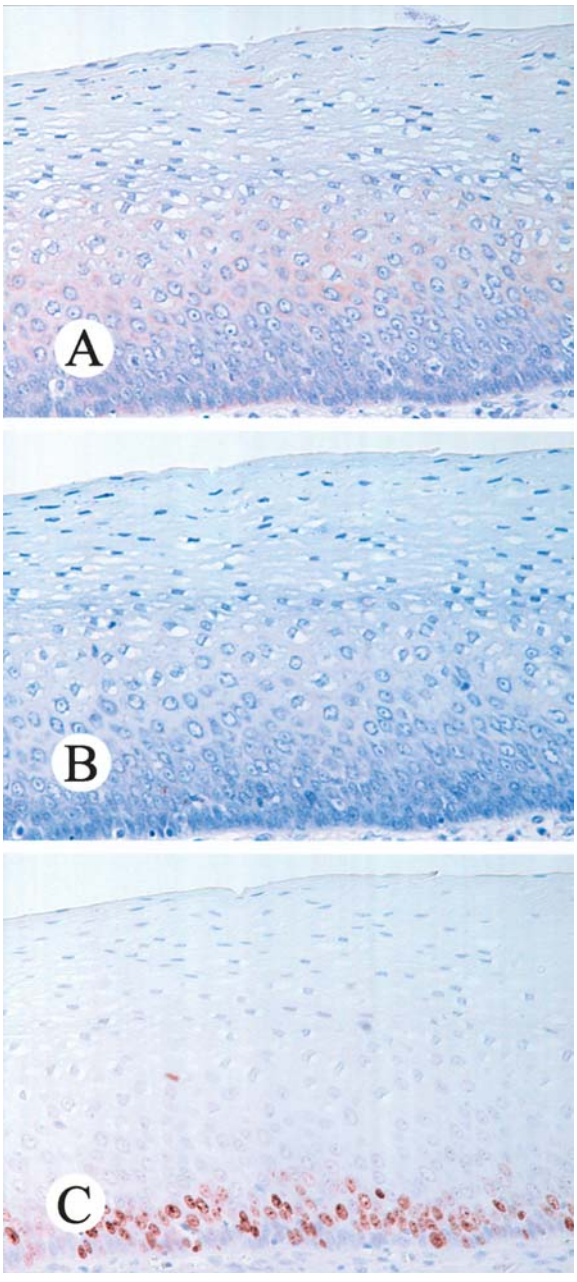

Figure 1. Immunohistochemical examination of KGFR, KGF and Ki-67 in noncancerous esophageal tissues. KGFR was localized in the basal region of the epithelium to the lower one-third of normal squamous epithelial cells (A). KGF was faintly localized in the basal region to the parabasal region (B). Ki-67 was localized in the parabasal region (C). Original magnification: x400.

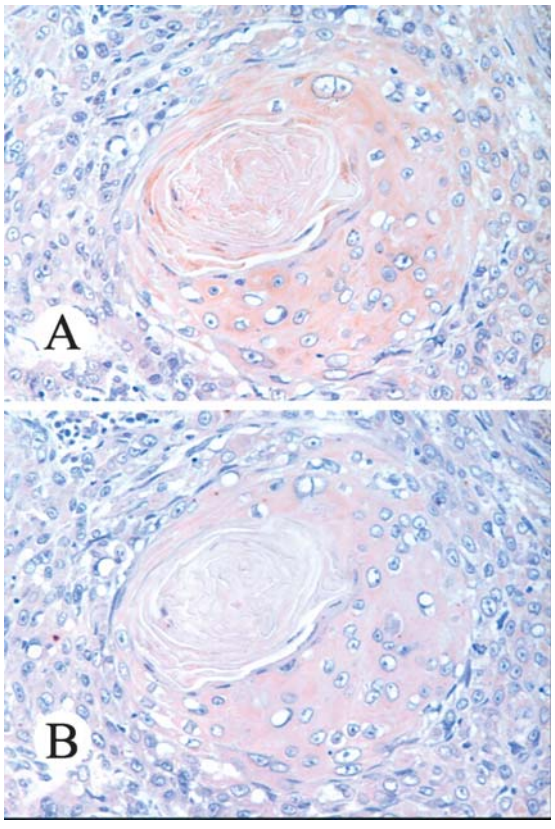

Figure 2. Characteristic staining pattern of coexpression of KGFR and KGF in esophageal cancer (EC) tissues. In squamous pearl of EC cells, KGFR and KGF were coexpressed (A and B, respectively). Original magnification: x400.

54 (26%) EC patients. Clinicopathologically, KGFR expression significantly correlated with well-differentiated cell types, but not with other factors ($p<0.001$, Table I). KGF expression significantly correlated with lymphatic invasion and lymph node metastasis ($p=0.004$ and 0.021, respectively, Table I). The coexpression of KGFR and KGF correlated with well-differentiated cell types ($p<0.001$).

Kaplan-Meier survival curve. The KGFR-positive, KGF-positive and KGFR/KGF coexpression patients tended to have shorter survival rates than the KGFR-, KGF- and KGFR/KGF-negative patients, but the survival rates were not statistically significantly different (Fig. 3A-C: $p=0.44$, 0.059 and 0.112, respectively).

RT-PCR of KGFR and KGF in EC cell lines. To examine the expression of KGFR and KGF in EC cell lines, RT-PCR analysis was performed. KGFR mRNA was detected in the TE-1, TE-8 and TE-11 cells (Fig. 4, upper panel), but no KGF mRNA was detected in any of the tested cancer cell lines (Fig. 4, middle panel). ß-actin mRNA was detected in all of the cancer cell lines tested (Fig. 4, lower panel).

Quantitative RT-PCR of KGFR in EC cell lines. To measure the expression level of KGFR mRNA in EC cell lines, qRT-PCR analysis was performed. The KGFR mRNA expression level was highest in the TE-1 cells and lowest in the TE-8 cells ($p<0.05$, Fig. 5). The KGFR mRNA expression level in the TE-1 cells was 6.4-fold higher than that in the TE-8 cells.

Western blot analysis of KGFR in EC cell lines. To characterize KGFR protein expression in EC cell lines, total protein was extracted from the TE-1, TE-8 and TE-11 cells. The Western blot analysis of these cell lines using the anti-KGFR antibody showed a single 105-kD band corresponding to KGFR (Fig. 6, upper panel). A 42-kD band corresponding to ß-actin was detected at almost the same intensities in all of the cancer cell lines (Fig. 6, lower panel).

Effect of rhKGF on EC cell growth. To determine whether KGF stimulates EC cell growth, rhKGF was added to the TE-1,

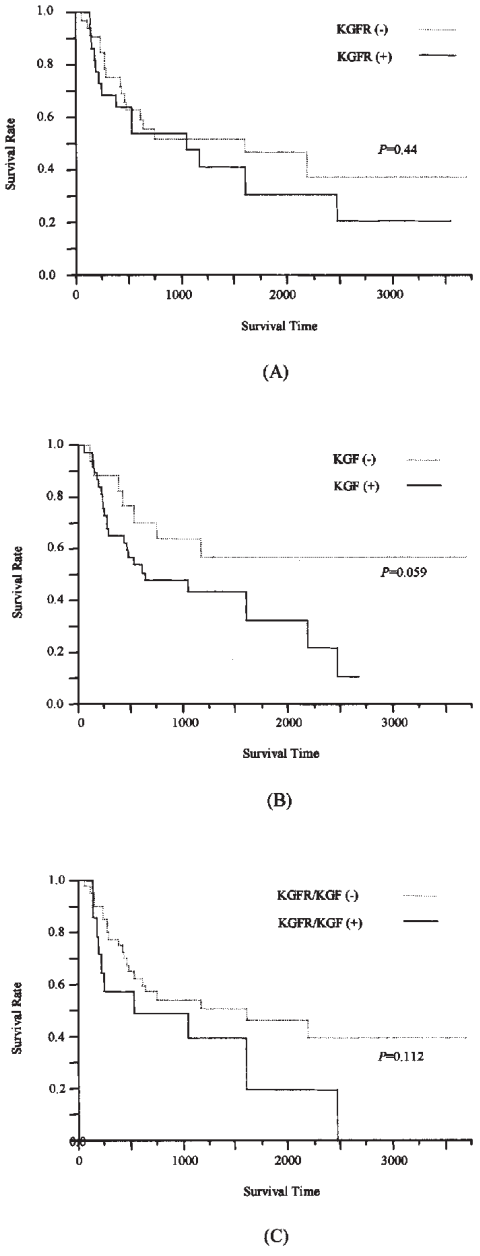

Figure 3. Kaplan-Meier survival curve analysis. Curves for all patients with KGFR-positive or KGFR-negative tumors (A). Curves for all patients with KGF-positive or KGF-negative tumors (B). Curves for all patients with presence or absence of both KGFR and KGF (C). No statistical significance was observed in all groups (A-C: p=0.44, 0.059 and 0.112, respectively).

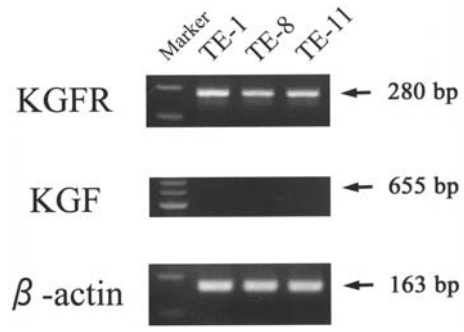

Figure 4. RT-PCR analyses of KGFR and KGF mRNAs in esophageal cancer (EC) cell lines. KGFR mRNA, corresponding to 280 bp, was detected in all three EC cell lines (upper panel); no KGF mRNA was detected in the cancer cell lines (middle panel). ß-actin mRNA was detected in all cancer cell lines (lower panel).

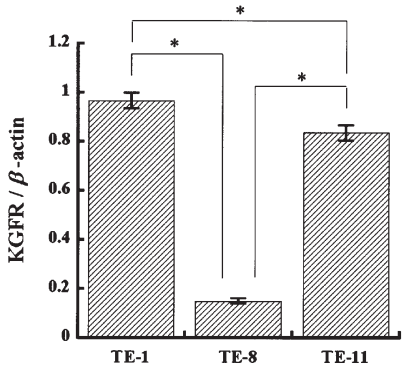

Figure 5. Quantitative RT-PCR analysis of KGFR mRNA expression level in esophageal cancer cell lines. The KGFR mRNA level was highest in the TE-1 cells and lowest in the TE-8 cells. *p<0.05.

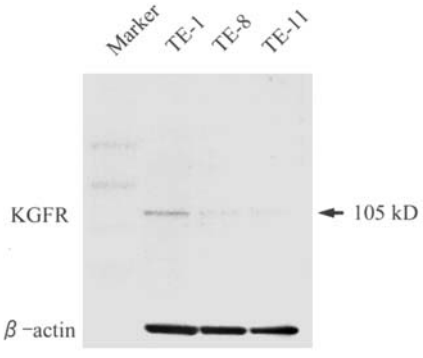

Figure 6. Western blot analysis of KGFR protein in esophageal cancer (EC) cell lines. Western blot analysis showed a single 105-kD band corresponding to the KGFR protein in EC cell lines (upper panel). A 42-kD band corresponding to the ß-actin protein was detected at almost equal intensity in the cancer cell lines (lower panel).

TE-8 and TE-11 cells. TE-1 did not proliferate with the addition of 10 or 100 ng/ml rhKGF (Fig. 7A, p=0.131 and 0.083, respectively). However, the growth rate of the TE-8 cells increased with the addition of 10 and 100 ng/ml KGF at 48 h, as compared with the nontreated controls (Fig. 7B, p=0.008 and 0.001, respectively). The growth rates of TE-11 cells increased with the addition of 10 and 100 ng/ml rhKGF (Fig. 7C, p=0.068 and 0.019, respectively).

Discussion

KGFR is localized in normal epithelial cells in the GI tract (9). In this study, we found that, in noncancerous esophageal tissues, KGFR is localized in the lower one-third of squamous epithelial cells and its localization is broader than that of Ki-67, a proliferating cell marker. In gastric and colorectal tissues, KGFR is localized in the luminal surface of epithelial cells, but Ki-67 is localized in the nuclei of ductal cells in the basal and parabasal regions (14,27). These findings suggest that KGFR plays important roles not only in epithelial cell proliferation in the GI tract, but also in cell differentiation.

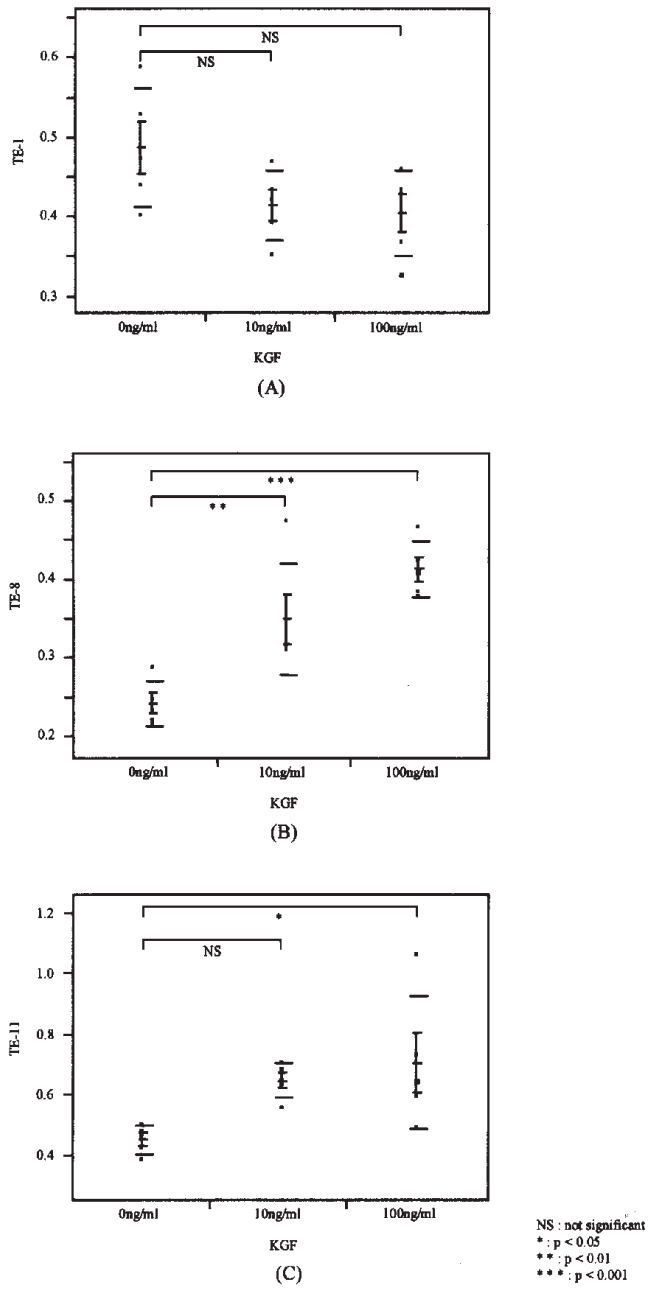

Figure 7. Effect of rhKGF on esophageal cancer cell growth. TE-1 growth was not significantly stimulated with the addition of 10 and 100 ng/ml rhKGF (A). In contrast, TE-8 and TE-11 cell growth rates increased after the addition of 10 and 100 ng/ ml rhKGF at 48 h (B and C). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; NS, not significant.

Histologically, most ECs are classified as SCC. In SCC, KGFR was expressed in uterine cervical cancer cell lines (26). KGFR-expressing uterine cervical cancer cells had a large pleomorphic cytoplasm and are histologically similar to well-differentiated SCC cells. Uterine cervical cancer cells showing a round or oval cytoplasm and a high nuclear/cytoplasm ratio as poorly differentiated SCC cells did not express KGFR. Previously, KGFR mRNA expression was reported to have been detected in 13 EC cell lines, but no KGF mRNA was present in the cells (18). Our results of KGFR and KGF mRNA expression in EC cells were identical with this report. All three EC cell lines that expressed KGFR originated from well-differentiated or moderately differentiated SCC cells. qRT-PCR analysis showed that KGFR mRNA is more strongly expressed in the TE-1 cells from well-differentiated SCC cells than in the TE-8 or TE-11 cells from moderately differentiated SCCs. These lines of evidence suggest that the KGFR expression level in cancer cells correlates with the well-differentiated type of SCC cells.

We previously reported the KGFR expression in cancer cell lines derived from the GI tract, including the esophagus, stomach and colon (14,27). Conventional gastric cancer cell lines expressed KGFR, but special types of gastric cancer cell line from metastatic foci of adenosquamous cells and α-fetoprotein-producing gastric cancer cells did not express KGFR (27,29). In colorectal cancer cell lines, KGFR was strongly expressed in cancer cells originating from well-differentiated adenocarcinoma (14). In human breast adenocarcinoma cell lines, the KGFR expression level in well-differentiated (estrogen receptor, ER-positive) cancer cell lines was markedly higher than in the poorly differentiated (ER-negative) cancer cell lines (30). KGFR, which is almost identical in molecular weight to KGFR in human colorectal cancer cell lines was detected in EC cell lines. To our knowledge, this is the first report on KGFR expression in EC cell lines at the protein level.

A previous report showed that KGF enhances DNA synthesis and induces TE-1 cell growth (18). In this study, KGF did not induce TE-1 cell growth, but induced TE-8 and TE-11 cell growth in a dose-dependent manner. The different responses of the TE-1 cells to KGF administration may depend on FBS concentration. We used 10% FBS in our culture medium, but previous researchers used a lower serum concentration of 0.01% or 0.7%. A higher FBS concentration contains many kinds of growth factors at high concentrations; thus, FBS may abolish the growth stimulatory effect of exogenous KGF in EC. Furthermore, the growth induction of KGF in the TE-8 and TE-11 cells at high FBS concentration suggests the high effectiveness of KGF on these cells. All these cells possess KGFR, but TE-1 cells have the highest KGFR expression level. Growth induction by rhKGF in SCC cells may not be dependent on KGFR mRNA expression level. Alternatively, a high KGFR expression level may have different effects, including those on TE-1 cell differentiation. KGF is reported to induce the growth of well-differentiated colorectal cells, but does not stimulate the growth of undifferentiated cancer cells (13,16). The TE-1 cells were from well-differentiated SCC cells, and the TE-8 and TE-11 cells were from moderately differentiated SCC cells. The reason for the different responses of these cells is not yet known; however, the role of KGF may differ between SCC and adenocarcinoma. Further study of the growth stimulatory effect of KGF on various histological types of cancer cell seems necessary.

Recently, we have found that KGF expression in pancreatic cancer cells correlates with venous invasion (28). In this study, exogenous KGF induced EC cell growth. Furthermore, KGF expression in EC cells correlates with lymphatic invasion and lymph node metastasis. EC cells whose growth has been induced by KGF may easily invade lymphatic vessels in the submucosa and lead to regional lymph node metastasis.

In summary, we found that KGFR is localized in a non-cancerous esophageal epithelium, and that KGFR expression

in EC correlates with highly differentiated histological type. Moreover, KGF has a potential to stimulate the growth of some EC cells and its expression correlates with lymphatic invasion and lymph node metastasis.

Acknowledgments

We express our gratitude to Dr Munehiko Onda (Department of Pathology, Nippon Medical School) for providing invaluable advice, and Ms Taeko Suzuki, Yoko Kawamoto and Kiyoko Kawahara and Mr Kiyoshi Teduka (Department of Pathology, Nippon Medical School) for excellent technical assistance. We also thank Ms Yuko Ono (Department of Pathology, Nippon Medical School) for assistance in manuscript preparation.

References

1. Itoh N and Ornitz DM: Evolution of the Fgf and Fgfr gene families. Trends Genet 20: 563-569, 2004.
2. Ornitz DM and Itoh N: Fibroblast growth factors. Genome Biol 2: Reviews S3005, 2001.
3. Miki T, Bottaro DP, Fleming TP, *et al*: Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. Proc Natl Acad Sci USA 89: 246-250, 1992.
4. Mohammadi M, Olsen SK and Ibrahimi OA: Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 16: 107-137, 2005.
5. Igarashi M, Finch PW and Aaronson SA: Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J Biol Chem 273: 13230-13235, 1998.
6. De Moerlooze L, Spencer-Dene B, Revest J, Hajihosseini M, Rosewell I and Dickson C: An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development 127: 483-492, 2000.
7. Revest JM, Spencer-Dene B, Kerr K, De Moerlooze L, Rosewell I and Dickson C: Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. Dev Biol 231: 47-62, 2001.
8. Xu X, Weinstein M, Li C, *et al*: Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development 125: 753-765, 1998.
9. Housley RM, Morris CF, Boyle W, *et al*: Keratinocyte growth factor induces proliferation of hepatocytes and epithelial cells throughout the rat gastrointestinal tract. J Clin Invest 94: 1764-1777, 1994.
10. Visco V, Belleudi F, Marchese C, *et al*: Differential response to keratinocyte growth factor receptor and epidermal growth factor receptor ligands of proliferating and differentiating intestinal epithelial cells. J Cell Physiol 200: 31-44, 2004.
11. Baatar D, Kawanaka H, Szabo IL, *et al*: Esophageal ulceration activates keratinocyte growth factor and its receptor in rats: implications for ulcer healing. Gastroenterology 122: 458-468, 2002.
12. Ishiwata T, Friess H, Buchler MW, Lopez ME and Korc M: Characterization of keratinocyte growth factor and receptor expression in human pancreatic cancer. Am J Pathol 153: 213-222, 1998.
13. Otte JM, Schmitz F, Banasiewicz T, Drews M, Folsch UR and Herzig KH: Expression of keratinocyte growth factor and its receptor in colorectal cancer. Eur J Clin Invest 30: 222-229, 2000.
14. Yoshino M, Ishiwata T, Watanabe M, *et al*: Keratinocyte growth factor receptor expression in normal colorectal epithelial cells and differentiated type of colorectal cancer. Oncol Rep 13: 247-252, 2005.
15. Siddiqi I, Funatomi H, Kobrin MS, Friess H, Buchler MW and Korc M: Increased expression of keratinocyte growth factor in human pancreatic cancer. Biochem Biophys Res Commun 215: 309-315, 1995.
16. Dignass AU, Tsunekawa S and Podolsky DK: Fibroblast growth factors modulate intestinal epithelial cell growth and migration. Gastroenterology 106: 1254-1262, 1994.
17. Nakazawa K, Yashiro M and Hirakawa K: Keratinocyte growth factor produced by gastric fibroblasts specifically stimulates proliferation of cancer cells from scirrhous gastric carcinoma. Cancer Res 63: 8848-8852, 2003.
18. Iida S, Katoh O, Tokunaga A and Terada M: Expression of fibroblast growth factor gene family and its receptor gene family in the human upper gastrointestinal tract. Biochem Biophys Res Commun 199: 1113-1119, 1994.
19. Oelmann E, Haghgu S, Kulimova E, *et al*: Influence of keratinocyte growth factor on clonal growth of epithelial tumor cells, lymphoma and leukemia cells and on sensitivity of tumor cells towards 5-fluorouracil *in vitro*. Int J Oncol 25: 1001-1012, 2004.
20. Ropiquet F, Huguenin S, Villette JM, *et al*: FGF7/KGF triggers cell transformation and invasion on immortalised human prostatic epithelial PNT1A cells. Int J Cancer 82: 237-243, 1999.
21. Zhang Y, Wang H, Toratani S, *et al*: Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis. Proc Natl Acad Sci USA 98: 11336-11340, 2001.
22. Spielberger R, Stiff P, Bensinger W, *et al*: Palifermin for oral mucositis after intensive therapy for hematologic cancers. N Engl J Med 351: 2590-2598, 2004.
23. Finch PW and Rubin JS: Keratinocyte growth factor expression and activity in cancer: implications for use in patients with solid tumors. J Natl Cancer Inst 98: 812-824, 2006.
24. Rosen LS, Abdi E, Davis ID, *et al*: Palifermin reduces the incidence of oral mucositis in patients with metastatic colorectal cancer treated with fluorouracil-based chemotherapy. J Clin Oncol 24: 5194-5200, 2006.
25. Ishiwata T, Naito Z, Lu YP, *et al*: Differential distribution of fibroblast growth factor (FGF)-7 and FGF-10 in L-arginine-induced acute pancreatitis. Exp Mol Pathol 73: 181-190, 2002.
26. Kurban G, Ishiwata T, Kudo M, Yokoyama M, Sugisaki Y and Naito Z: Expression of keratinocyte growth factor receptor (KGFR/FGFR2 IIIb) in human uterine cervical cancer. Oncol Rep 11: 987-991, 2004.
27. Matsunobu T, Ishiwata T, Yoshino M, *et al*: Expression of keratinocyte growth factor receptor correlates with expansive growth and early stage of gastric cancer. Int J Oncol 28: 307-314, 2006.
28. Cho K, Ishiwata T, Uchida E, *et al*: Enhanced expression of keratinocyte growth factor and its receptor correlates with venous invasion in pancreatic cancer. Am J Pathol (in press).
29. Hattori Y, Itoh H, Uchino S, *et al*: Immunohistochemical detection of K-sam protein in stomach cancer. Clin Cancer Res 2: 1373-1381, 1996.
30. Zang XP and Pento JT: Keratinocyte growth factor-induced motility of breast cancer cells. Clin Exp Metastasis 18: 573-580, 2000.