The American Journal of Pathology, Vol. 170, No. 6, June 2007

DOI: 10.2353/ajpath.2007.060935

Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Enhanced Expression of Keratinocyte Growth Factor and Its Receptor Correlates with Venous Invasion in Pancreatic Cancer

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Keratinocyte growth factor (KGF) and KGF receptor (KGFR) have been implicated in cancer growth as well as tissue development and repair. In this study, we examined whether KGF and KGFR have a role in human pancreatic ductal adenocarcinoma (PDAC). KGFR mRNA was expressed in eight pancreatic cancer cell lines, whereas the KGF mRNA was detected in seven of the cell lines and was absent in MIA PaCa-2 cells. KGFR and KGF immunoreactivity were localized in the cancer cells in 41.5 and 34.0% of patients, respectively. There was a significant correlation between KGFR or KGF immunoreactivity and venous invasion and a significant correlation between the presence of both markers and venous invasion, vascular endothelial growth factor (VEGF)-A expression, and poor prognosis. Exogenous KGF increased VEGF-A expression and release in MIA PaCa-2 cells, and PANC-1 cells stably transfected to overexpress KGF-exhibited increased VEGF-A expression. Moreover, short hairpin-KGFR transfection in MIA PaCa-2 cells reduced the stimulatory effect of exogenous KGF on VEGF-A expression. Short hairpin-KGF transfection in KLM-1 cells reduced VEGF-A expression in the cells. KGFR and KGF may act to promote venous invasion and tumor angiogenesis in PDAC, raising the possibility that they may serve as novel therapeutic targets in anti-angiogenic strategies in PDAC. ***(Am J Pathol 2007, 170:1964–1974; DOI: 10.2353/ajpath.2007.060935)***

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with few long-term survivors. Although the management and treatment of patients with PDAC have improved in the last few decades, the overall 5-year survival rate remains at less than 5%, underscoring the need for more effective therapeutic approaches. Clinically, this poor prognosis occurs because PDAC is often diagnosed at a late stage when the cancer is no longer resectable. Moreover, those patients that undergo resection frequently exhibit a high incidence of local recurrence, lymph node metastasis, hepatic metastasis, and peritoneal dissemination. At the molecular level, PDAC often harbors mutations in the K-*ras* oncogene, the p53 tumor suppressor gene, and the p16 cell cycle gene.[1] A high percentage of PDACs also overexpress a number of growth factors and their receptors, including the epidermal growth factor (EGF) receptor, EGF, transforming growth factor-α, CRIPTO, transforming growth factor-β1, basic fibroblast growth factor (FGF), acidic FGF, and FGF-5.[2–7] The multiple alterations in oncogenes and tumor suppressor genes in conjunction with the overexpression of mitogenic growth factors and their receptors may contribute to the biological aggressiveness of pancreatic cancers and to the formation of the abundant stroma that is characteristic of this malignancy.[5,6]

Keratinocyte growth factor (KGF) is a member of the FGF group of heparin-binding polypeptides that was originally isolated from human embryonic lung fibroblasts.[8,9] KGF is synthesized by mesenchymal cells and T lymphocytes and acts predominantly on epithelial cells in a paracrine manner.[10,11] It shares 30 to 70% amino acid sequence homology with other FGFs. In addition to KGF, which is also known as FGF-7, this family includes acidic FGF or FGF-1, basic FGF or FGF-2, int-2 (FGF-3), hst/K-FGF (FGF-4), FGF-5, FGF-6, androgen-induced growth factor (FGF-8), glia activating factor (FGF-9), FGF-10, FGF-11 (FGF homologous factors-3), FGF-12 (FGF homologous factors-1), FGF-13 (FGF homologous fac-

Supported by a grant-in-aid for Scientific Research (C, no. 17591445) from the Japan Society for the Promotion of Science.

Accepted for publication March 14, 2007.

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tors-2), FGF-14 (FGF homologous factors-4), and FGF-16 to FGF-23.[8,9,12,13] KGF actions are dependent on its binding to a specific cell-surface KGF receptor (KGFR), also known as FGF receptor (FGFR) type II (FGFR-2IIIb).[14] This receptor possesses intrinsic tyrosine kinase activity and binds KGF and FGF-1 with high affinity but does not bind FGF-2.[14] The extracellular domain of KGFR consists of two or three immunoglobulin-like (Ig-like) regions, whereas its intracellular domain contains a tyrosine kinase region that is interrupted by a nonkinase intervening sequence.[15] KGFR is encoded by the *fgfr-2* gene.[15] Because FGFR-2IIIc and KGFR derive from the same gene, the two receptors are homologous in their intracellular domains and most of their extracellular domains. However, they differ from each other in the carboxyl-terminal half of the third Ig-like region of the extracellular domain, as a consequence of alternative mRNA splicing.[15] FGFR-2IIIc is mainly localized in mesenchymal cells, whereas KGFR is localized in epithelial cells.

KGF is expressed in a variety of tissues including the lung, prostate, mammary gland, digestive tract, bladder, and skin and is implicated in organ development and homeostasis.[10,16,17] KGF expression is dramatically upregulated in cutaneous wounds, where it can speed the repair process, and also in the hyperplastic skin disease psoriasis.[18–20] KGF also stimulates the growth of hair follicles and the thickening of the gastrointestinal tract mucosa.[21,22] Moreover, KGF-expressing transgenes exhibit pancreatic ductal hyperplasia, and KGF mRNA levels are elevated in PDAC.[21,23–25] Although we previously reported that KGFR and KGF were overexpressed in both the pancreatic cancer cells and the adjacent pancreatic parenchyma,[26] the potential roles of KGFR and KGF in PDAC are still poorly understood. We now report that the coexpression of KGFR and KGF in PDAC is associated with a propensity for venous invasion, enhanced vascular endothelial growth factor (VEGF)-A expression, and poor prognosis.

Materials and Methods

Materials

The chemicals and reagents were purchased as follows: Isogen from Nippon Gene (Tokyo, Japan); a Takara RNA PCR kit (AMV) version 3.0 and pBAsi-hU6 Neo DNA vector from Takara Biotech (Tokyo, Japan); RNeasy mini kit from Qiagen GmbH (Hilden, Germany); Transcriptor First Strand cDNA Synthesis kit and LightCycler FastStart DNA Master SYBR Green I, FuGENE 6, and FuGENE HD transfection reagent from Roche Diagnostics GmbH (Mannheim, Germany); human VEGF Quantikine Colorimetric Sandwich enzyme-linked immunosorbent assay (ELISA) kit, goat polyclonal anti-FGF-7 antibodies, and recombinant human KGF (rhKGF) from R&D Systems Inc. (Westerville, OH); Immobilon P transfer membrane from Millipore (Yonezawa, Japan); M-PER Mammalian Protein Extraction reagent and Super Signal West Pico chemiluminescent substrates from Pierce (Rockford, IL); SERVA Blau G from Serva Electrophoresis GmbH (Heidelberg, Germany); Histofine Simple Stain Max PO (G) or (R) kit from Nichirei Biosciences, Inc. (Tokyo, Japan); anti-rabbit IgG-horseradish peroxidase secondary antibody and rabbit polyclonal anti-VEGF-A antibodies (A-20) from Santa Cruz Biotechnology (Santa Cruz, CA); Human Tissue Microarray 1 and Human Digestive Tissue Sets from Novagen (Darmstadt, Germany); fluorescein 5-isothiocyanate-conjugated anti-rabbit IgG and Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole dihydrochloride from Vector Laboratories, Inc. (Burlingame, CA); silane-coated slides and a malinol mounting medium from Muto Pure Chemicals Co., Ltd. (Tokyo, Japan); and pIRES2-EGFP vector from Clontech (Palo Alto, CA). All other chemicals and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

Patients and Tissues

Tissues from 53 patients with invasive PDAC were obtained for this study. These patients received treatment at Nippon Medical School Hospital (Bunkyo-ku, Tokyo, Japan) from 1995 to 2003. None of the patients received preoperative chemotherapy and radiotherapy. The patients consisted of 36 males and 17 females, whose median age was 64 years (range, 35 to 84 years). The clinicopathological stage was determined according to the TNM classification system of the International Union Against Cancer[27] and additionally characterized with the Japan Pancreas Society classification[28] (Table 1). Thirty-one patients did not receive postoperative chemotherapy, and 22 patients received adjuvant chemotherapy after surgery. Twelve patients received Uracil/Tegafur, and 10 patients received gemcitabine. The median follow-up period was 14.1 months. Paraffin-embedded specimens were prepared for immunohistochemical analysis as described previously.[26] This study was performed in accordance with the principles embodied in the Declaration of Helsinki 1975, and informed consent for the usage of pancreatic tissues was obtained from each patient. Normal pancreatic tissues were obtained from Human Digestive Tissue Sets and Human Tissue Microarray 1.

Pancreatic Cancer Cell Lines

PANC-1, MIA PaCa-2, KLM-1, and PK-1, -8, -9, and -59 pancreatic ductal adenocarcinoma cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), and Capan-1 was purchased from American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 200 U/ml penicillin, and 200 μg/ml kanamycin at 37°C under a humidified 5% CO_2 atmosphere. Capan-1 was grown in the same medium containing 15% FBS.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from pancreatic cancer cell lines using Isogen according to the manufacturer's pro-

Table 1. Correlation of Clinicopathological Features and KGFR, KGF, or Coexpression of KGFR and KGF in Pancreatic Cancers

Variables	*n*	KGFR		KGF		KGFR and KGF	
		n (%)	*P*	*n* (%)	*P*	*n* (%)	*P*
Gender							
Male	36	17 (47)	NS	12 (33)	NS	9 (25)	NS
Female	17	5 (29)		6 (35)		4 (24)	
Age							
<65	24	8 (33)	NS	7 (29)	NS	3 (13)	NS
65+	29	14 (48)		11 (38)		10 (34)	
UICC classification							
T-primary tumor							
T1	3	2 (67)	NS	1 (33)	NS	1 (33)	NS
T2	3	1 (33)		1 (33)		1 (33)	
T3	15	7 (47)		8 (53)		5 (33)	
T4	32	12 (36)		8 (25)		6 (19)	
N-Regional lymph nodes							
N0	19	11 (58)	NS	8 (42)	NS	6 (32)	NS
N1	34	11 (32)		10 (29)		7 (21)	
M-Distant metastasis							
M0	51	21 (41)	NS	17 (33)	NS	12 (24)	NS
M1	2	1 (50)		1 (50)		1 (50)	
G-Histological grading							
G1	31	14 (45)	NS	8 (26)	NS	6 (19)	NS
G2	21	7 (33)		9 (43)		6 (29)	
G3	1	1 (100)		1 (100)		1 (100)	
G4	0	0		0		0	
Stage							
I or II	10	5 (50)	NS	1 (10)	NS	4 (40)	NS
III or IV	43	17 (40)		17 (40)		9 (21)	
Other tumor characteristics							
Lymphatic invasion							
Negative	6	3 (50)	NS	2 (33)	NS	2 (33)	NS
Positive	47	19 (40)		16 (34)		11 (23)	
Venous invasion							
Negative	35	11 (31)	0.038	8 (23)	0.017	5 (14)	0.016
Positive	18	11 (61)		10 (56)		8 (44)	
Nerve invasion (intrapancreatic)							
Negative	11	6 (55)	NS	5 (45)	NS	3 (27)	NS
Positive	42	16 (38)		13 (31)		10 (24)	

UICC, International Union Against Cancer; NS, not significant.

tocol. Then, cDNA synthesis and polymerase chain reaction (PCR) were performed using the Takara RNA PCR kit. The primer pair used for KGFR corresponded to nucleotides 1587 to 1606 (5′-CACTCGGGGATAAATAGTTC-3′) and nucleotides 1719 to 1736 (5′-CGCTTGCTGTTTTG-GCAG-3′) (150 bp, accession no. NM022970). The primers used for KGF corresponded to nucleotides 765 to 784 (5′-TTGTGGCAATCAAAGGGGTG-3′) and nucleotides 905 to 927 (5′-CCTCCGTTGTGTGTCCATTTAGC-3′) of the human KGF cDNA (163 bp, accession no. NM00209). β-Actin mRNA, as the positive control, was amplified using the following primer pairs: nucleotides 331 to 353 (5′-GCACCACACCTTCTACAATGAGC-3′) and nucleotides 472 to 493 (5′-TAGCACAGCCTGGATAGCAACG-3′) (163 bp, accession no. NM001101). The authenticity of the PCR product was confirmed by the direct sequence method. Total RNA not subjected to reverse transcription was used as the negative control.

Quantitative Real-Time PCR

Total RNA extraction from tumor cells was performed using the RNeasy Mini kit. cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis kit following the manufacturer's protocol. Quantitative real-time PCR (Q-PCR) was performed using a LightCycler-FastStart DNA Master SYBR Green I system. The same KGFR, KGF, and β-actin primer pairs used for reverse transcription-PCR (RT-PCR) were used for real-time PCR analysis. PCR reaction mixture containing 2 μl of template cDNA, 3 mmol/L $MgCl_2$, and 0.5 μmol/L of primers, and LightCycler-FastStart DNA Master SYBR Green I mix was applied into a capillary tube (Roche). Q-PCR was performed in a LightCycler (Roche), and the PCR products were analyzed by LightCycler Data Analysis software version 3.5 (Roche). The optimized program involved denaturation at 95°C for 10 minutes, followed by 50 cycles of amplification as follows: for KGFR, at 95°C for 10 seconds, at 60°C for 10 seconds, and at 72°C for 7 seconds; for KGF, at 95°C for 10 seconds, at 58°C for 10 seconds, and at 72°C for 8 seconds; and for β-actin, at 95°C for 10 seconds, at 64°C for 10 seconds, and at 72°C for 7 seconds. To confirm amplification specificity, PCR products were subjected to a melting-curve analysis. Results were expressed as target/β-actin as an internal standard concentration ratio. Gene expression measurements were performed in triplicate.

Western Blot Analysis

Protein extraction was performed according to the protocol involving the use of the M-Per Mammalian Protein Extraction reagent. Briefly, the cultured pancreatic cancer cells were solubilized in M-Per reagent with Protease Inhibitor Cocktail for Mammalian Tissues. Lysates were centrifuged for 10 minutes at 13,000 rpm to pellet cell debris. The supernatants were collected, and protein concentration was measured by the Bradford method. The anti-KGFR antibody used in this study was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids of the human KGFR protein.[25] The cleared protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and the separated proteins were transferred to Immobilon P transfer membranes, which were then incubated for 16 hours at 4°C with the anti-KGFR antibody. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody for 60 minutes. After washing, the blot was visualized by enhanced chemiluminescence.

Immunofluorescence Staining and Confocal Laser Microscopy

The same anti-KGFR antibody used for the Western blot analysis was used for immunofluorescence staining of MIA PaCa-2 cells. MIA PaCa-2 cells were incubated with the anti-KGFR antibody (1:100) in PBS containing 1% bovine serum albumin for 16 hours at 4°C. For negative control, MIA PaCa-2 cells were incubated with PBS containing 1% bovine serum albumin. The cells were washed with PBS and then incubated with fluorescein 5-isothiocyanate-conjugated anti-rabbit IgG. One hour after incubation, the cells were washed with PBS and then mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole dihydrochloride. Fluorescent images were acquired using a Digital Eclipse TE 2000-E confocal laser scanning microscope (Nikon Insteck Co., Ltd., Tokyo, Japan) and a 100× immersion lens (Nikon Palm Apo VC) with blue diode and argon lasers and were analyzed using the confocal microscope and Digital Eclipse C1 control software EZ-C1 (version 2.30) (Nikon Insteck). The excitation wavelength for fluorescein 5-isothiocyanate was 488 nm, and emission was selected and recorded using a 500- to 530-nm band-pass filter. In addition, the excitation wavelength for 4′,6-diamidino-2-phenylindole dihydrochloride was 405 nm, and emission was selected and recorded using a 432- to 446-nm band-pass filter.

Immunohistochemistry

Paraffin-embedded tissue sections (3.5 μm) were subjected to immunostaining using the Histofine Simple Stain Max PO (G) or (R) kit. After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 minutes, and the sections were incubated with the appropriate antibody for 16 hours at 4°C (1:1000 dilution for the anti-KGFR antibody, 1:50 dilution for the anti-KGF antibody, and 1:200 dilution for the anti-VEGF-A antibody) using PBS containing 1% bovine serum albumin. The anti-KGFR antibody used was the same antibody used in Western blot analysis and immunofluorescence staining. Bound antibodies were detected with Simple Stain Max PO (G) or (R) reagents using diaminobenzidine-tetrahydrochloride as the substrate, and the sections were counterstained with Mayer's hematoxylin. Negative control studies were performed by omitting the primary antibodies. The immunohistochemical results for KGFR, KGF, and VEGF-A were evaluated as follows: when staining was noted in the cytoplasm and/or membrane of more than 30% of the tumor cells, regardless of the intensity of staining, the cells were designated as positive. Two investigators (K.C. and T.I.) separately evaluated all of the specimens in a blinded manner.

Effects of rhKGF on VEGF-A mRNA Expression

MIA PaCa-2 cells (1×10^5/well), plated in six-well plates, were grown in 2 ml of RPMI 1640 medium with 10% FBS for 24 hours and then cultured with serum-free medium for 48 hours. The cells were subsequently cultured in serum-free RPMI 1640 medium in the absence or presence of 10 ng/ml rhKGF for 1, 3, 6, and 12 hours. Expression of VEGF-A and RS-18 mRNA levels were examined by Q-PCR as previously described. The real-time PCR primers used for VEGF-A corresponded to nucleotides 1126 to 1148 (5′-GAGGAGGGCAGAATCATCACGAA-3′) and nucleotides 1348 to 1369 (5′-TGGTGAGGTTTGATCCGCATAA-3′) of the human VEGF-A cDNA (244 bp, accession no. NM003376). The primers used for RS-18 corresponded to nucleotides 184 to 207 (5′-AAAGCAGACATTGACCTCACCAAG-3′) and nucleotides 319 to 341 (5′-AGGACCTGGCTGTATTTTACC-3′) of the human RS-18 cDNA (158 bp, accession no. NM022551). The same KGF primer pairs used for RT-PCR were used for real-time PCR analysis. The optimized program involved denaturation at 95°C for 10 minutes, followed by 45 cycles of amplification at 95°C for 10 seconds, at 60°C for 10 seconds, and at 72°C for 10 seconds for VEGF-A; at 95°C for 10 seconds, at 65°C for 10 seconds, and at 72°C for 7 seconds for RS-18; and at 95°C for 10 seconds, at 58°C for 10 seconds, and at 72°C for 8 seconds for KGF. To confirm amplification specificity, PCR products were subjected to melting-curve analysis. Results were expressed as target/RS-18, as an internal standard concentration ratio. Each experiment was performed twice with gene expression measurements performed in triplicate.

Effects of rhKGF on VEGF-A Protein Levels

MIA PaCa-2 cells (2×10^3/well) were plated in 96-well plates, grown in 200 μl of RPMI 1640 medium with 10% FBS for 24 hours, and then cultured with serum-free medium for 48 hours. The cells were subsequently cultured in serum-free RPMI 1640 medium with 0, 10, or 100

ng/ml rhKGF for 48 hours. The culture supernatants were collected, and their VEGF-A levels were measured using ELISA kits. This study was performed by two separate experiments, each conducted in triplicate.

KGF and VEGF-A mRNA Levels in KGF Transfected PANC-1 Cells

Full-length KGF cDNA fragment was prepared by RT-PCR from human placental RNA. The primer pair used for the cDNA was 5′-GCTAGCAAGGAGATACCACCATG-CACAAATGGATACTGACAT-3′ (including the *Nhe*I site) and 5′-CTGCAGCAATTAAGTTATTGCCATAG-3′ (including the *Pst*I site). The full-length cDNA was digested with *Nhe*I and *Pst*I and ligated to the 3′ end of the human cytomegalovirus early promoter/enhancer in pIRES2-EGFP eukaryotic expression vector. Proper insert orientation was verified by DNA sequencing. pIRES2-EGFP contains the internal ribosome entry site of the encephalomyocarditis virus between the KGF and the enhanced green fluorescent protein coding region. This permits both the KGF and the enhanced green fluorescent protein genes to be translated from a single bicistronic mRNA. Approximately 1×10^6/ml PANC-1 cells were transfected with 2 μg of DNA using FuGENE 6, and the cells were passaged and cultured with 500 μg/ml G-418. Independent colonies were isolated by ring cloning, transferred to micro titer wells, and expanded. KGF stably transfected PANC-1 cells (PANC-1-KGF) and mock-transfected PANC-1 cells (PANC-1-Mock) (1×10^5/well) were plated in six-well plates and grown in 2 ml of RPMI 1640 medium with 10% FBS for 24 hours and then cultured with serum-free medium for 72 hours. Expression of KGF and VEGF-A mRNA levels were examined by Q-PCR as previously described.

Construction of Short Hairpin-KGFR and Short Hairpin-KGF

To construct vectors for human short hairpin (sh)-KGFR and KGF, a DNA fragment flanked by the *Bam*HI and *Hin*dIII sites containing the sense target sequence for KGFR (5′-GATAAATAGTTCCAATGCA-3′), the hairpin loop sequence (5′-TAGTGCTCCTGGTTG-3′), and the antisense target sequence was synthesized and inserted into pBAsi-hU6 Neo DNA vector. The sense target sequence for KGF is 5′-GAAGTTATGATTACATGGA-3′. Likewise, the control sequence for KGFR (5′-GATA-GAATCGACTATAACT-3′) and the control sequence for KGF (5′-GGAATGATGCTATATGTAA-3′) were used to construct the vectors for negative controls (sh-control).

Transient Transfection

Twenty-four hours before transfection, MIA PaCa-2 cells (5×10^5/dish) were plated in 100-mm dishes and grown in 7 ml of RPMI 1640 medium with 10% FBS. Transfections of sh-KGFR and sh-control were performed using FuGENE HD transfection reagent, according to the manufacturer's instructions. To confirm the effective transfection of sh-KGFR in MIA PaCa-2 cells, cell lysates were collected, and KGFR protein levels were measured by Western blot analysis. Forty-eight hours after transfection, the culture medium from MIA PaCa-2 cells was replaced with serum-free medium containing 0 or 10 ng/ml rhKGF, and incubations were continued for 48 hours. The culture supernatants were collected, and VEGF-A levels were measured using the ELISA kit. KLM-1 cells (2×10^5/well) were seeded in six-well plates and grown for 24 hours in 2 ml of RPMI 1640 medium with 10% FBS. Transfections of sh-KGF and sh-control were performed as described above. The medium from KLM-1 cells was replaced with a serum-free medium 48 hours after transfection, and cells were incubated 48 hours. The culture supernatants were collected, and their VEGF-A levels were measured using ELISA kits. The mRNA levels for KGF and VEGF-A in KLM-1 cells were measured by Q-PCR. All shRNA experiments were conducted twice, with triplicate determinations per experiment.

Statistical Analysis

Whenever indicated, the χ^2 test and Fisher's exact test were used to analyze the correlation between KGFR, KGF, or VEGF-A expression and clinicopathological features. Cumulative survival rate was calculated by the Kaplan-Meier method, and the significance of differences in survival rate was analyzed by the log-rank test. $P < 0.05$ was considered significant in all analyses. Computations were performed using the StatView J version 5.0 software package (SAS Institute, Inc., Cary, NC).

Results

RT-PCR and Q-PCR Analysis of KGFR and KGF Expression in Pancreatic Cancer Cell Lines

Expression of KGFR and KGF was examined in PANC-1; MIA PaCa-2; KLM-1; Capan-1; and PK-1, -8, -9, and -59 cells. A 150-bp band corresponding to KGFR mRNA was detected in all pancreatic cancer cell lines (Figure 1A, top panel). By contrast, a 163-bp band corresponding to KGF mRNA was detected in all but one of the pancreatic cancer cell lines, with MIA PaCa-2 cells not exhibiting the transcript (Figure 1A, middle panel). A 163-bp band corresponding to β-actin mRNA was used as a loading control and was detected in all cancer cell lines (Figure 1A, bottom panel). Likewise, Q-PCR analysis was performed to quantify KGFR and KGF mRNA expression levels in pancreatic cancer cell lines. KGFR mRNA was present at variable levels in all of the pancreatic cancer cell lines (Figure 1B). KGF mRNA was not detected in MIA PaCa-2 cells but was expressed at variable levels in the other pancreatic cancer cell lines (Figure 1C).

Western blot analysis was performed next to assess the expression of KGFR protein in the cancer cell lines. A 105-kd band corresponding to KGFR and a 42-kd band corresponding to β-actin were detected in all pancreatic cancer cell lines (Figure 1D, top and bottom panels,

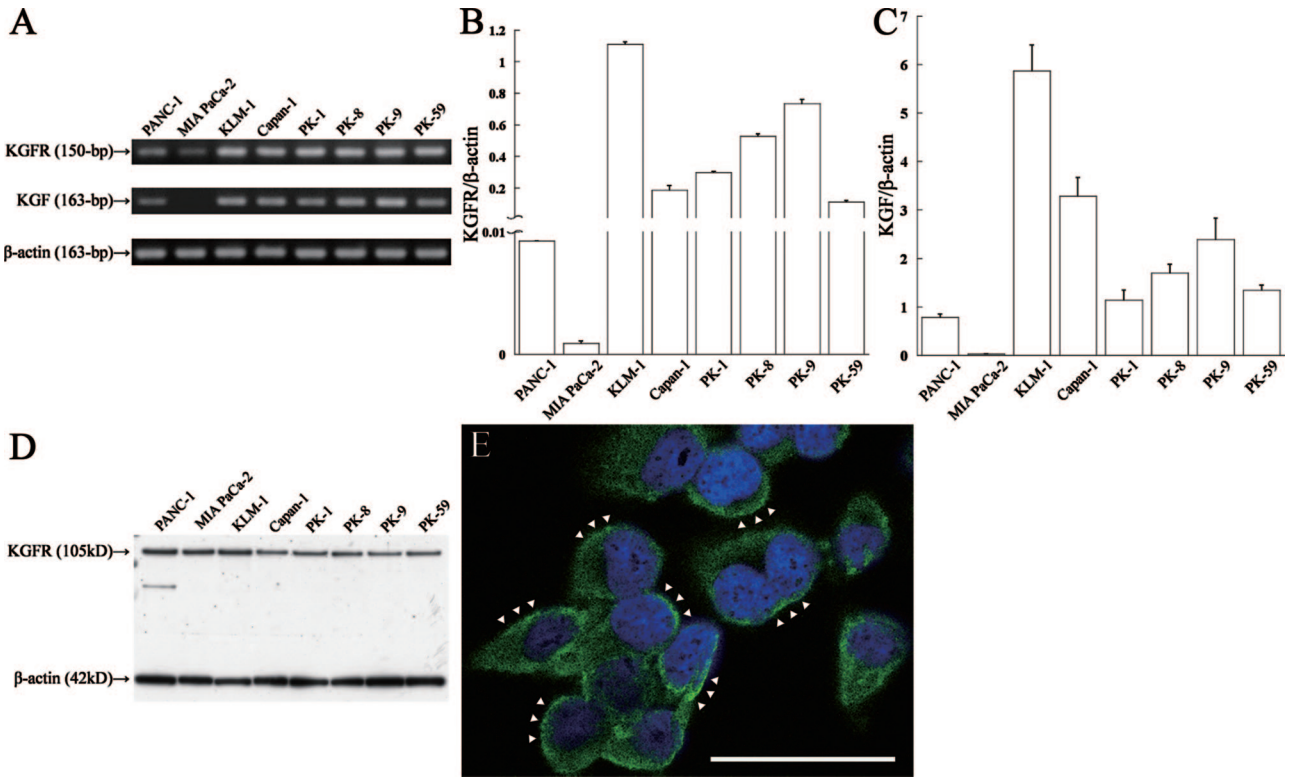

Figure 1. KGFR and KGF in pancreatic cancer cell lines. **A:** RT-PCR analysis of KGFR and KGF mRNA in pancreatic cancer cell lines. Total RNA was extracted from PANC-1; MIA PaCa-2; KLM-1; Capan-1; and PK-1, -8, -9, and -59 cells and used to perform cDNA synthesis and PCR analysis. KGFR mRNA (150 bp) was detected in all cell lines (top). KGF mRNA (163-bp) was detected in all pancreatic cancer cell lines, except for MIA PaCa-2 cells (middle). β-Actin mRNA (163 bp) served as a loading control (bottom). **B** and **C:** Q-PCR analysis of KGFR and KGF mRNA in pancreatic cancer cell lines. KGFR mRNA was expressed at variable levels in all pancreatic cancer cell lines. KGF mRNA was not detected in MIA PaCa-2 cells and was expressed at variable levels in the other pancreatic cancer cell lines. Results are expressed as KGFR/β-actin and KGF/β-actin. Gene expression measurements were performed in triplicate. Bars represent the mean ± SE. **D:** Western blot analysis of KGFR in pancreatic cancer cell lines. A band corresponding to the 105-kd KGFR protein was detected in all pancreatic cancer cell lines (top). β-Actin served as a loading control (bottom). **E:** Immunofluorescent analysis of KGFR expression in MIA PaCa-2 cells. KGFR immunoreactivity was detected in the cytoplasm and/or on the membrane of cancer cells. In some cases, a strong KGFR signal was detected at the cell membrane (**arrowheads**). Bar = 50 μm.

respectively). To confirm expression of KGFR at the protein level, immunofluorescent analysis using confocal laser microscopy was performed next. This analysis revealed that KGFR protein was present in the cytoplasm and the cell membrane of MIA PaCa-2 cells, with some cells exhibiting a strong KGFR signal at the cell membrane (Figure 1E, arrowheads). KGFR signals were not detected in negative control cells without anti-KGFR antibody (data not shown).

Immunohistochemical Analysis of KGFR and KGF in Pancreatic Cancer Tissues

Immunohistochemical analysis of the PDAC samples was performed next to determine whether there was a correlation between KGFR and KGF expression in the cancer cells and the clinicopathological features. KGFR was localized in the cytoplasm and/or membrane of the cancer cells in 22 of the 53 (41.5%) patients (Figure 2A), whereas KGF immunoreactivity was detected in cytoplasm of the cancer cells in 18 of the 53 (34.0%) patients (Figure 2B). There was a statistically significant correlation between immunoreactivity for either KGFR (P = 0.038) or KGF (P = 0.017) and the presence of venous invasion (Table 1). Moreover, the concomitant expression of KGFR and KGF was observed in 13 of the 53 (24.5%) samples, and this concomitant expression also correlated with venous invasion (P = 0.016; Table 1). By contrast, in normal pancreatic tissues, KGFR immunoreactivity was detected in the cytoplasm and/or membrane of islet cells and, to a lesser extent, pancreatic ductal cells. KGF was also present in the islet cells, as well as in a few stromal fibroblasts, pancreatic ductal cells, and acinar cells (data not shown), as previously reported.[26] In the chronic pancreatitis-like lesions adjacent to the cancer cells, expression of KGFR and KGF were more strongly observed in the same cell types as in the normal pancreas (data not shown).

Immunohistochemical Analysis of VEGF-A in Pancreatic Cancer Tissues

Because VEGF-A is a major angiogenic growth factor in PDAC,[29] we next sought to determine whether coexpression of KGFR and KGF correlated with VEGF-A expression. In the normal pancreas, VEGF-A was occasionally detected in the cytoplasm of pancreatic ductal cells, islet cells, and acinar cells (data not shown). In the PDAC samples, VEGF-A immunoreactivity was observed in cytoplasm of the cancer cells in 19 of the 53 (35.8%) pa-

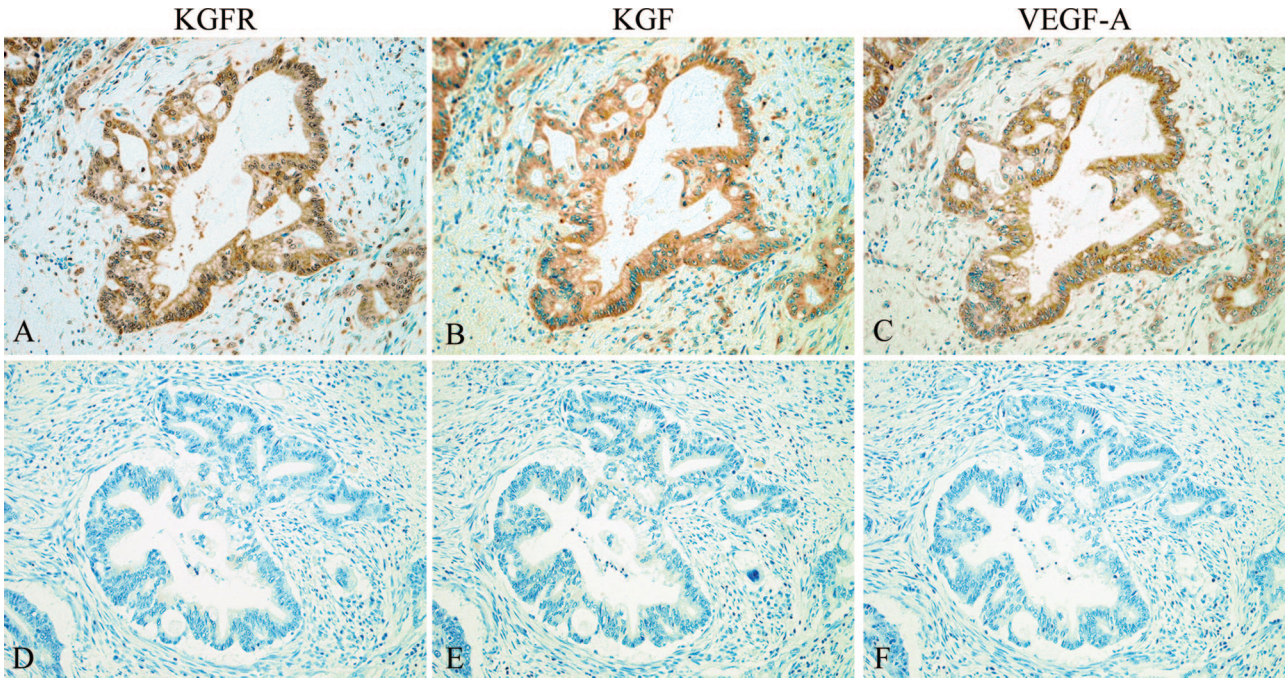

Figure 2. Immunohistochemical analyses of KGFR, KGF, and VEGF-A in human pancreatic cancer using serial tissue sections. **A–C:** Characteristic staining patterns of KGFR, KGF, and VEGF-A in human pancreatic cancer cases. **A:** KGFR immunoreactivity was detected in the cytoplasm and cell membrane of cancer cells. **B:** KGF immunoreactivity was detected in the cytoplasm of cancer cells and stromal fibroblasts. **C:** VEGF-A immunoreactivity was detected in the cytoplasm of cancer cells and stromal fibroblasts. **D–F:** KGFR-, KGF-, and VEGF-A-negative cases. Immunohistochemistry, KGFR (**A** and **D**), KGF (**B** and **E**), and VEGF-A (**C** and **F**); original magnification, ×200.

tients (Figure 2C). The presence of VEGF-A in the cancer cells correlated with the presence of KGFR ($P = 0.017$), KGF ($P = 0.032$), or both ($P = 0.026$; Table 2).

Cumulative Kaplan-Meier Survival Curve and Multivariate Analysis

The overall 2-year survival rate of all 53 cases of PDAC was 17.0%. The survival rates of the KGFR-positive group and KGFR-negative group were not significantly different ($P = 0.33$; Figure 3A). However, the survival rate of the KGF-positive group was significantly shorter than that of the KGF-negative group ($P = 0.017$; Figure 3B). Furthermore, the survival rate of the PDAC patients whose cancer cells were positive for both KGFR and KGF was also significantly shorter than those of patients whose cancers did not express either marker ($P = 0.014$; Figure 3C). Next, the survival rates of the 31 patients that had surgery but did not receive any adjuvant chemotherapy were analyzed in a similar manner. In these patients, there was no statistically significant difference between the KGFR-positive or the KGF-positive groups and the corresponding negative groups (Figure 3, D and E). By contrast, the survival rate for the surgery-only patients whose tumors were both KGFR and KGF positive was significantly shorter than the corresponding negative group ($P = 0.033$; Figure 3F). Furthermore, multivariate analysis for overall survival using Cox's proportional hazard model was performed in relation to gender, age, tumor stage, KGFR expression, KGF expression, VEGF-A expression, and coexpression of KGFR and KGF in both the surgery-only group ($n = 31$) and in all of the patients ($n = 53$). Only coexpression of KGFR and KGF showed a statistically significant result either in the surgery-only group (risk ratio, 15; $P = 0.039$; Table 3) or in all of the patients (risk ratio, 7.5; $P = 0.028$; Table 3). Thus, the coexpression of KGFR and KGF was an independent prognostic factor.

Table 2. Correlation of VEGF-A Expression and KGFR, KGF, or Coexpression of KGFR and KGF

KGFR/KGF expression	VEGF-A expression: Negative ($n = 34$)	VEGF-A expression: Positive ($n = 19$)	P
KGFR expression			
Negative ($n = 31$)	24	7	0.017
Positive ($n = 22$)	10	12	
KGF expression			
Negative ($n = 35$)	26	9	0.032
Positive ($n = 18$)	8	10	
Coexpression of KGFR and KGF			
Negative ($n = 40$)	29	11	0.026
Positive ($n = 13$)	5	8	

Effect of KGF on VEGF-A Expression in Pancreatic Cancer Cells

Our immunostaining and survival results pointed to a close relationship between the KGF/KGFR pathway and VEGF-A. Therefore, three different types of experiments were performed next to assess the relationship between the KGF/KGFR pathway and VEGF-A. In the first set of

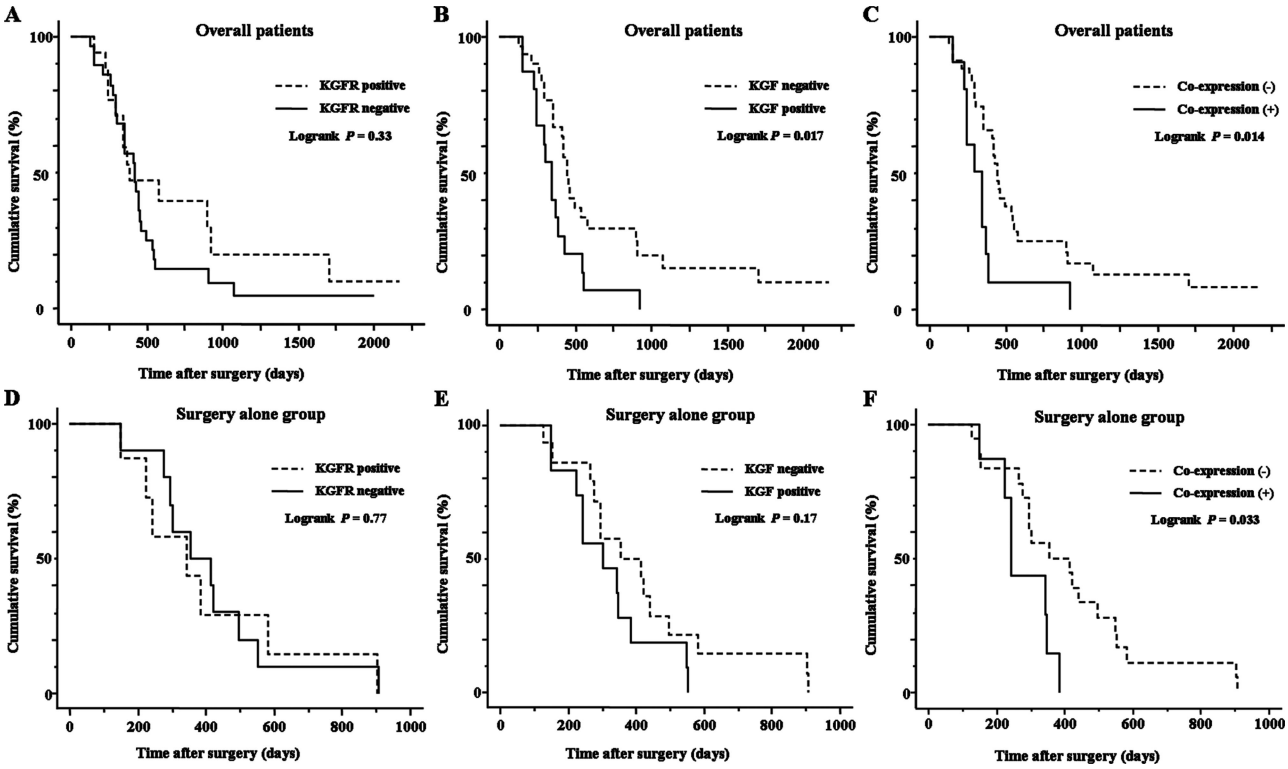

Figure 3. Cumulative Kaplan-Meier survival curves. **A:** Curves for all patients with KGFR-positive or -negative tumors. **B:** Curves for all patients with KGF-positive or -negative tumors ($P = 0.017$). **C:** Curves for all patients with the presence or absence of both KGFR and KGF ($P = 0.014$). **D–F:** Curves for the subgroup of patients who underwent surgery alone. Statistical significance was only observed in the patients whose tumors were positive for both KGFR and KGF ($P = 0.033$; **F**).

experiments, MIA PaCa-2 cells, which are KGFR-positive and KGF-negative by RT-PCR and Q-PCR analysis, were incubated for up to 6 hours in the absence or presence of rhKGF (10 ng/ml), and VEGF-A mRNA levels were determined by Q-PCR. rhKGF caused a significant increase in VEGF-A mRNA levels (Figure 4A). Furthermore, VEGF-A protein levels in the conditioned medium were significantly increased after incubation with either 10 or 100 ng/ml rhKGF ($P = 0.034$ and 0.018, respectively; Figure 4B). These results indicated that exogenous KGF can induce VEGF-A expression in pancreatic cancer cells.

Table 3. Prognostic Factors in Multivariate Analysis by Cox's Proportional Hazard Risk Model

Variable	95% confidence		
	Risk ratio	Interval	*P*
Surgery alone group ($n = 31$)			
Gender	1.6	0.53–4.9	0.39
Age (years)	1.0	0.97–1.1	0.46
Stage	1.1	0.51–2.4	0.79
KGFR	5.2	0.57–47	0.14
KGF	0.74	0.18–3.1	0.68
VEGF-A	0.69	0.20–2.4	0.55
Coexpression of KGFR and KGF	15	1.1–189	0.039
Overall patients ($n = 53$)			
Gender	1.4	0.61–3.4	0.41
Age (years)	1.0	0.98–1.1	0.34
Stage	1.6	0.87–2.9	0.13
KGFR	3.7	0.96–14	0.058
KGF	1.6	0.53–5.0	0.40
VEGF-A	1.9	0.76–4.5	0.17
Coexpression of KGFR and KGF	7.5	1.2–46	0.028

In a second set of experiments, we examined the effect of endogenously produced KGF on VEGF-A expression. To this end, we compared VEGF-A expression in PANC-1-Mock cells with VEGF-A expression in PANC-1-KGF. As expected, Q-PCR revealed that KGF mRNA levels were significantly higher in PANC-1-KGF cells than in PANC-1-Mock cells ($P < 0.0001$; Figure 4C). Moreover, by Q-PCR, VEGF-A mRNA levels were significantly higher in PANC-1-KGF cells than in the PANC-1-Mock cells ($P = 0.004$; Figure 4D).

Finally, to establish a better link between KGF/KGFR pathway and VEGF-A expression, we performed experiments using sh-KGFR and sh-KGF. sh-KGFR and sh-control were transiently transfected into MIA PaCa-2 cells, and their ability to down-regulate expression levels of VEGF-A protein was tested by the addition of rhKGF (10 ng/ml). sh-KGFR significantly reduced KGFR protein levels in MIA PaCa-2 cells compared with sh-control, as determined by Western blot analysis ($P = 0.0006$; Figure 4E). These cells also exhibited significantly reduced VEGF-A protein levels in the conditioned medium in response to rhKGF (10 ng/ml) compared with sh-control transfected cells ($P = 0.0016$; Figure 4F). Next, sh-KGF and sh-control were transiently transfected into KLM-1 cells that expressed the highest KGF levels among all of the pancreatic cancer cell lines. Q-PCR revealed that

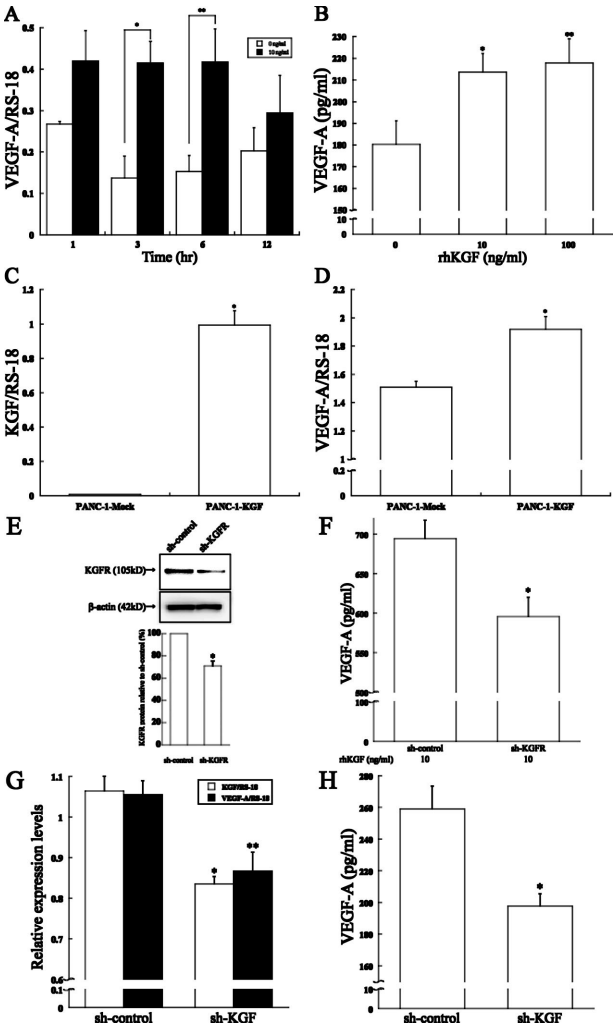

Figure 4. Effect of KGF on VEGF-A expression in pancreatic cancer cells. **A:** Time course of VEGF-A induction. Q-PCR analysis showed significant increases in VEGF-A mRNA levels at 3 and 6 hours after the addition of 10 ng/ml rhKGF to MIA PaCa-2 cells. Each experiment was performed twice, and gene expression measurements were performed in triplicate. Bars represent the mean ± SE (*$P = 0.038$, **$P = 0.041$). **B:** Effects of exogenous KGF on VEGF-A protein levels. After the addition of rhKGF (0 to 100 ng/ml) to MIA PaCa-2 cells, VEGF-A levels in the culture supernatant were measured by ELISA and were found to be increased significantly in a dose-dependent manner. Results shown represent the mean ± SE of two separate experiments, each conducted in triplicate (*$P = 0.034$, **$P = 0.018$). **C** and **D:** Effects of engineered expression of KGF on VEGF-A expression. PANC-1-KGF and PANC-1-Mock cells were incubated in serum-free medium for 72 hours. KGF and VEGF-A mRNA levels were then determined by Q-PCR analysis. KGF (*$P < 0.0001$; **C**) and VEGF-A (*$P = 0.004$; **D**) mRNA levels in PANC-1-KGF were significantly higher than in PANC-1-Mock cells. Each experiment was performed twice, and each measurement was performed in triplicate. Bars represent the mean ± SE. **E** and **F:** Effects of sh-KGFR on VEGF-A expression. Western blot analysis showed that sh-KGFR inhibited the expression of KGFR protein levels in MIA PaCa-2 cells compared with sh-control (*$P = 0.0006$; **E**). Cells were treated with rhKGF (10 ng/ml) after being transfected with sh-KGFR or sh-control. sh-KGFR transfection was associated with significantly reduced VEGF-A protein levels in the conditioned medium after the addition of rhKGF (10 ng/ml) compared with sh-control (*$P = 0.0016$; **F**). Each measurement was performed in triplicate. Bars represent the mean ± SE. **G** and **H:** Effects of sh-KGF on VEGF-A expression. sh-KGF and sh-control were transiently transfected into KLM-1 cells. Q-PCR revealed that sh-KGF significantly reduced KGF and VEGF-A mRNA levels in KLM-1 cells compared with sh-control (*$P = 0.0002$, **$P = 0.0046$; **G**). Furthermore, sh-KGF significantly reduced VEGF-A protein levels in conditioned medium compared with sh-control (*$P = 0.0016$; **H**). Each experiment was performed twice, and each measurement was performed in triplicate. Bars represent the mean ± SE.

sh-KGF significantly reduced KGF and VEGF-A mRNA levels in KLM-1 cells compared with sh-control ($P = 0.0002$ and 0.0046, respectively; Figure 4G). Moreover, sh-KGF significantly reduced VEGF-A protein levels in the conditioned medium compared with sh-control ($P = 0.0016$; Figure 4H). These results clearly demonstrate that a KGF/KGFR signaling pathway is implicated in VEGF-A regulation.

Discussion

The FGFR family of transmembrane tyrosine kinase receptors and its 23 ligands participate in the modulation of many physiological processes regulating proliferation, migration, survival, and differentiation.[30,31] Moreover, as a result of the existence of multiple FGF ligands, and four distinct FGFR genes that encode four major FGFRs that yield many splice variants,[12] there is a tremendous potential for cross talk and cross-modulation in this receptor family. Therefore, proper tissue-specific expression of FGFs and FGFRs is of critical importance in assuring normal FGFR signaling.[30,32] For example, KGF is a highly efficient ligand for KGFR and does not bind other FGF receptors. Restriction of KGFR expression to epithelial cells means that KGF actions are also restricted to such cells. Such cell type-specific localization assures proper epithelial-mesenchymal interactions required for normal tissue function and organogenesis.[33] The importance of regulating FGFR2 isoform expression is underscored by the observation that there is a switch in expression from FGFR2(IIIb) to FGFR2(IIIc) during the progression of prostate carcinomas.[34]

In fetal pancreatic tissues, KGF induces β-cell expansion through the activation of ductal cell proliferation and their subsequent differentiation into β-cells.[35] Moreover, transgenic mice expressing KGF showed significant intra-islet ductal cell proliferation in pancreas.[36] In the normal human pancreas, KGF was mostly present in the islet cells, whereas KGFR was present both in the islet and ductal cells.[26] In some studies of cancer cell lines, KGFR mRNA was shown to be expressed in LNCaP, ND-1, and DU-145 prostatic cancer cell lines, but KGF mRNA was not detected.[37] By contrast, using more sensitive PCR techniques, KGFR mRNA was detected in five of seven pancreatic cancer cell lines, and KGF was expressed in four of these cell lines. In one of three pancreatic cancer cell lines, KGF induced the growth of cancer cells, but KGF did not stimulate the growth other tested cell lines (ASPC-1 and PANC-1 cells).[24] Furthermore, KGFR and KGF were strongly expressed in cancer cells of human pancreatic cancer tissues.[26] Taken together, these observations suggest that there is aberrant mis-expression of KGFR and KGF in human pancreatic cancer, suggesting that both KGFR and KGF may have a role in this malignancy.

In the present study, we determined that KGFR mRNA was expressed in eight pancreatic cancer cell lines and that KGFR and KGF mRNA were coexpressed in seven of the eight cancer cell lines. KGFR mRNA was translated to 105-kd KGFR protein in all cancer cell lines, as evi-

denced by immunoblotting. This is the first report of KGFR protein expression in pancreatic cancer cell lines. KGFR protein was localized by immunofluorescent staining on the surface of the cultured pancreatic cancer cells, as well as in the cytoplasm. These findings indicated that KGFR and KGF are coexpressed in pancreatic cancer cell lines and that at KGFR may be present at relatively high levels in these cells.

To delineate more clearly the role of KGFR and KGF in pancreatic cancer, we next sought to determine whether there was a correlation between KGFR and/or KGF expression and clinicopathological findings in pancreatic cancer. KGFR and KGF were detected in 41.5 and 34.0% of 53 human pancreatic cancer samples, and their coexpression was detected in 24.5% of the samples. The presence of either KGFR or KGF or the coexpression of KGFR and KGF correlated with the presence of venous invasion. Furthermore, there was a significant correlation between the presence of KGF or the coexpression of KGFR and KGF and poor prognosis. By contrast, KGFR expression in pancreatic cancer cells was not associated with a poor prognosis. Survival analysis was also performed in the 31 patients who underwent surgery but who opted not to receive adjuvant chemotherapy. This analysis revealed that the coexpression of KGFR and KGF was associated with poor prognosis. Likewise, multivariate analysis for overall survival using Cox's proportional hazard model revealed that the coexpression of KGFR and KGF was an independent prognostic factor. These findings suggest that the KGFR/KGF pathway may contribute to venous invasion, thereby resulting in a worse prognosis for pancreatic cancer patients. In partial support of this conclusion, previous studies have shown that KGFR and KGF mRNA were co-overexpressed in colorectal cancer cells[38] and that the coexpression of KGFR and KGF correlated with tumor proliferative activity, lymph node metastasis, and shorter 5-year survival in lung adenocarcinoma cases.[39]

Any individual tumor may have dominant angiogenic factors that induce angiogenesis by favoring an imbalance between positive and negative regulators. These angiogenic regulators act either directly on endothelial cells or indirectly by inducing the production of other regulators. Among these factors, VEGF-A is a potent angiogenic factor that clearly acts on endothelial cells in a direct manner.[40] Recently, it has been reported that enhanced VEGF-A expression correlates with hematogenous metastasis and prognosis in human colon, gastric, and pancreatic cancers.[41–43] Moreover, VEGF-A expression was correlated with blood vessel number, large tumor size, and enhanced local spread.[44] In addition to expressing high levels of VEGF-A, pancreatic cancers overexpress many pro-angiogenic factors. These include VEGF-C, transforming growth factor-α, and related EGF-like ligands (FGF-1, -2, and -5; hepatocyte growth factor; transforming growth factor-β1, -2, and -3; interleukin 8; and platelet-derived growth factor B).[29] KGF can also directly induce endothelial cell proliferation.[45] Moreover, in the present study, we determined that expression of KGFR or KGF or coexpression of KGFR and KGF in human pancreatic cancer tissues correlated with VEGF-A expression. Taken together, these observations suggest that KGF may induce angiogenesis in pancreatic cancer through a direct effect on endothelial cells or indirectly by inducting VEGF-A.

In the present study, we also determined that both exogenous and endogenous KGF up-regulates VEGF-A expression in pancreatic cancer cells. Moreover, sh-KGFR and sh-KGF significantly reduced VEGF-A expression in pancreatic cancer cells. Previous studies have shown that various cytokines including FGF-2, EGF, platelet-derived growth factor, interleukin 6, and transforming growth factor-β1 as well as oxidative stress and hypoxia induce VEGF-A expression[46–49] and that KGF induces VEGF expression in airway epithelial cell lines *in vitro*.[50] Taken together, these findings suggest that KGF synthesized by pancreatic cancer may also play an important role in enhancing venous invasion in pancreatic cancer, in part, by up-regulating VEGF-A expression. Inasmuch as venous invasion is a bad prognostic marker, our findings also suggest that KGFR and KGF may represent important targets that could allow for the development of novel therapeutic strategies to improve the survival of pancreatic cancer patients.

Acknowledgments

We express our appreciation to Dr. Mitsuhiro Kudo, Takenori Fujii, Kiyoshi Teduka, Yoko Kawamoto, Taeko Suzuki, and Kiyoko Kawahara (Department of Integrative Pathology, Nippon Medical School) for their excellent technical assistance. We also thank Dr. Yuichi Sugisaki (Division of Surgical Pathology, Nippon Medical School Hospital) for preparing tissue blocks.

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