Keratinocyte growth factor stimulates growth of MIA PaCa-2 cells through extracellular signal-regulated kinase phosphorylation

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Abstract. Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7, is mainly synthesized by mesenchymal cells. KGF modulates proliferation, differentiation, migration and adhesion to extracellular matrices of epithelial cells that specifically express the KGF receptor (KGFR). We previously reported that KGF is expressed in cancer cells and adjacent stromal fibroblasts in human pancreatic cancer tissues. Furthermore, KGF is thought to stimulate the growth of certain pancreatic cancer cell lines. The aim of the present study was to examine whether the mitogen-activated protein kinase (MAPK) pathway contributes to exogenous KGF-induced pancreatic cancer cell growth. Recombinant human KGF (rhKGF) was administered to MIA PaCa-2 cells, which expressed KGFR and negligible levels of KGF. Cell growth rates in MIA PaCa-2 cells were significantly increased in a dose-dependent manner following the addition of rhKGF. In the MAPK pathway, phosphorylation of extracellular signal-regulated kinase (ERK) in MIA PaCa-2 cells was increased in a dose-dependent manner, and phosphorylation of p38 was slightly increased following the administration of 100 ng/ml rhKGF. In contrast, JNK was not phosphorylated following the addition of rhKGF in MIA PaCa-2 cells. U0126, a specific inhibitor of ERK activation, decreased the rhKGF-induced phosphorylation of ERK and the growth rates of MIA PaCa-2 cells. These findings indicated that phosphorylation of the ERK signaling pathway plays a significant role in exogenous KGF-induced pancreatic cancer cell growth.

Introduction

Keratinocyte growth factor (KGF), also known as fibroblast growth factor (FGF)-7, was initially identified in a human embryonic lung fibroblast cell line (1,2). KGF is produced and secreted from various types of mesenchymal cells, including fibroblasts, smooth muscle cells and endothelial cells (2). KGF transcription is up-regulated in mesenchymal cells by platelet-derived growth factor (3,4) and only acts on KGF receptor (KGFR) expressed on epithelial cells (5,6). The KGFR is tyrosine kinase FGF receptor-2 (FGFR-2) IIIb, a spliced variant of FGFR2 (7). KGF modulates proliferation, differentiation, migration and adhesion to extracellular matrices of epithelial cells (8,9). KGF plays significant roles in the wound healing of skin, proliferation of gut epithelium and angiogenesis in the rat cornea (10).

In the healthy human pancreas, KGF is localized in the islet cells, whereas KGFR is present in the islet and ductal cells (11). Previously, we reported that KGF was strongly expressed in the cancer cells of 34% of pancreatic ductal adenocarcinoma (PDAC) patients (12). KGF expression in the cancer cells was significantly correlated with shorter survival (12). KGF was also strongly expressed in the fibroblasts adjacent to cancer cells and the chronic pancreatitis-like lesions adjacent to cancer cells in PDAC patients (11-13). A small number of studies using pancreatic cancer cell lines revealed that KGF stimulated pancreatic cancer cell growth. Recombinant human KGF (rhKGF) induced the growth of one of three PDAC cells, termed COLO 357 (14). rhKGF also induced the growth of HPAF-II, a metastatic pancreatic cancer cell line (15). These findings indicated that endogenous and exogenous KGF may contribute to PDAC cell growth and a poor prognosis. However, the signaling pathway involved in KGF-induced pancreatic cancer cell growth is not fully understood.

The aim of the present study was to examine whether the mitogen-activated protein kinase (MAPK) pathway, including the extracellular signal-regulated kinase (ERK), p38 and JNK pathways, contributes to exogenous KGF-induced pancreatic cancer cell growth. We report that KGF-induced activation of the ERK signaling pathway plays a significant role in pancreatic cancer cell growth.

Materials and methods

Materials. The following were purchased from the indicated suppliers: rhKGF from R&D Systems, Inc. (Westerville, OH, USA); WST-8 cell counting kit from Wako Pure Chemical Industries (Osaka, Japan); rabbit polyclonal anti-ERK-1 antibody, rabbit polyclonal anti-phospho-p38 antibody, rabbit polyclonal anti-p38 antibody and rabbit polyclonal anti-JNK

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antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit monoclonal anti-phospho-p44/42 MAPK antibody and mouse monoclonal anti-phospho-SAPK/JNK antibody from Cell Signaling Technology Inc. (Beverly, MA, USA); HRP-conjugated goat anti-rabbit IgG secondary antibody and HRP-conjugated goat anti-mouse IgG secondary antibody from American Qualex (San Clemente, CA, USA); M-PER Mammalian Protein Extraction Reagent and Super Signal West Dura Extended Duration Substrate from Thermo Fisher Scientific, Inc. (Waltham, MA, USA); Immobilon-P transfer membrane from Millipore (Tokyo, Japan) and U0126 from Merck Biosciences (Bad Soden, Germany). Other chemicals and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO, USA).

PDAC cell line. The human PDAC cell line MIA PaCa-2 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were grown in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37˚C under a humidified 5% CO_2 atmosphere.

Effect of rhKGF on cell growth of PDAC cells. MIA PaCa-2 cells ($1x10^5$ cells/well) were plated in 6-well plates and grown in 2 ml of RPMI-1640 with 10% FBS for 24 h. The cultured medium was then changed to serum-free medium in the presence or absence of 10 or 100 ng/ml rhKGF for 48 h. The cells were then incubated with WST-8 cell counting reagent for 4 h at 37˚C and the optical density of the culture solution in the plate was measured using an ELISA plate reader (Bio-Rad Laboratory) at 450 nm.

Western blot analysis. The MIA PaCa-2 cells were grown in RPMI-1640 with 10% FBS for 24 h and cultured with serum-free medium for 24 h. To determine the effect of KGF on the signaling pathway for cell proliferation, 10 or 100 ng/ml rhKGF was added to the culture medium and cells were incubated for 30 min. The cells were then solubilized in M-PER Mammalian Protein Extraction Reagent with Protease Inhibitor Cocktail for Mammalian Tissues. The protein concentration of the cell extract was measured by the Bradford method. Equal concentrations of the cell extract of each sample were subjected to SDS-PAGE under reducing conditions, and the separated proteins were transferred to Immobilon-P transfer membranes. The membranes were immersed for 2 h at room temperature (RT) in 5% skim milk in Tris-buffered saline containing 0.05% Tween-20, and incubated with an anti-phospho-p44/42 MAPK antibody (dilution, 1:1000), anti-phospho-p38 antibody (dilution, 1:1000), or anti-phospho-SAPK/JNK antibody (dilution, 1:1000) at 4˚C overnight. The membranes were washed and incubated with the HRP-conjugated anti-rabbit IgG antibody (dilution, 1:4000) or HRP-conjugated anti-mouse IgG antibody (dilution, 1:4000) for 60 min at RT. After washing, the blots were visualized using enhanced chemiluminescence and detected with a ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). To ensure similar amounts of protein in each sample, the same membranes were reprobed with anti-ERK-1 antibody (dilution, 1:1000), anti-p38 antibody (dilution, 1:1000) or anti-JNK antibody (dilution, 1:1000) and developed with HRP-conjugated secondary antibody using Super Signal West Dura Extended Duration Substrate. Three independent experiments were performed.

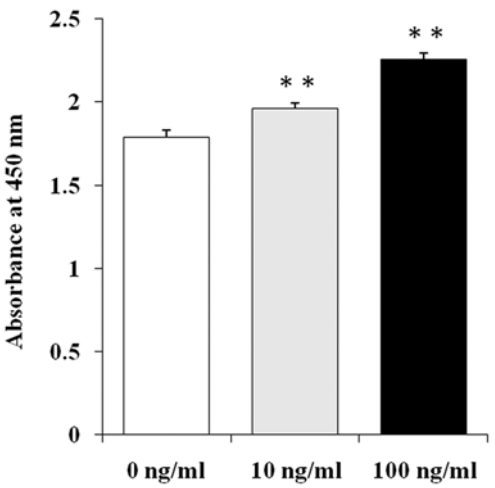

Figure 1. Cell growth assay of KGF-administered MIA PaCa-2 cells. Cells were cultured for 48 h in the presence or absence of 10 or 100 ng/ml rhKGF, and then incubated with WST-8 cell counting reagent. KGF-treated MIA PaCa-2 cells showed significantly higher growth rates, which occurred in a dose-dependent manner, than the untreated cells. $^{**}p<0.01$.

Effect of inhibition of the ERK signaling pathway on KGF-induced cell proliferation. MIA PaCa-2 cells ($1x10^5$ cells/well) were grown in RPMI-1640 with 10% FBS for 24 h, and then cultured with serum-free medium for 24 h. Prior to KGF administration (100 ng/ml), the cells were cultured with U0126 (10 mM) for 30 min. The cells were then cultured for 48 h and the cell growth rate was measured as described above.

Statistical analysis. Data were expressed as the mean ± SE. Data between the groups were compared using the Mann-Whitney U test. $P<0.05$ was considered to be significant in all analyses. Statistical analysis was performed using StatView Ver. 5.0.1 software (SAS Institute Inc, NC, USA).

Results

Cell growth assay of rhKGF-treated MIA PaCa-2 cells. MIA PaCa-2 cells, which express KGFR and negligible levels of KGF (KGFR-positive, KGF-negligible), were used to examine the effect of exogenous KGF on pancreatic cancer cell growth (12). After the addition of KGF, the growth rates of the MIA PaCa-2 cells were significantly increased compared to the untreated control cells. The growth stimulatory effects of KGF on the MIA PaCa-2 cells occurred in a dose-dependent manner at 10 and 100 ng/ml KGF (Fig. 1, $p<0.01$).

Effect of rhKGF on the MAPK signaling pathway in MIA PaCa-2 cells. We then examined phosphorylation of the MAPK signaling pathway, including the ERK, p38 and JNK pathways, to determine the signaling pathway induced by exogenous KGF in MIA PaCa-2 cells. The phosphorylated level of ERK in MIA PaCa-2 cells was increased in a dose-dependent manner after the addition of rhKGF (Fig. 2, top panel, p-ERK). Conversely, p38 was moderately activated by the addition of

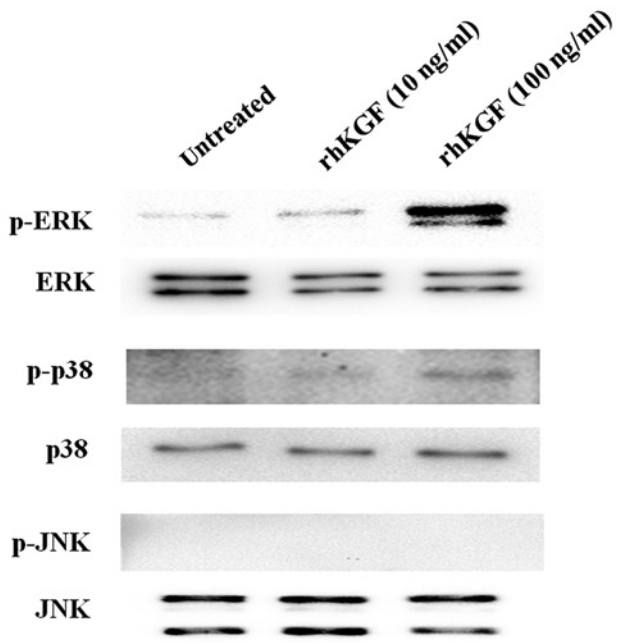

Figure 2. Effects of rhKGF on the MAPK signaling pathway in MIA PaCa-2 cells. The phosphorylation of ERK was increased in a dose-dependent manner by the addition of rhKGF (top panel, p-ERK). p38 was moderately activated by the addition of 100 ng/ml rhKGF (third panel from top, p-p38), and JNK was not activated by the addition of rhKGF (fifth panel from top, p-JNK). The second, fourth, and bottom panels show the total ERK, total p38 and total JNK, respectively.

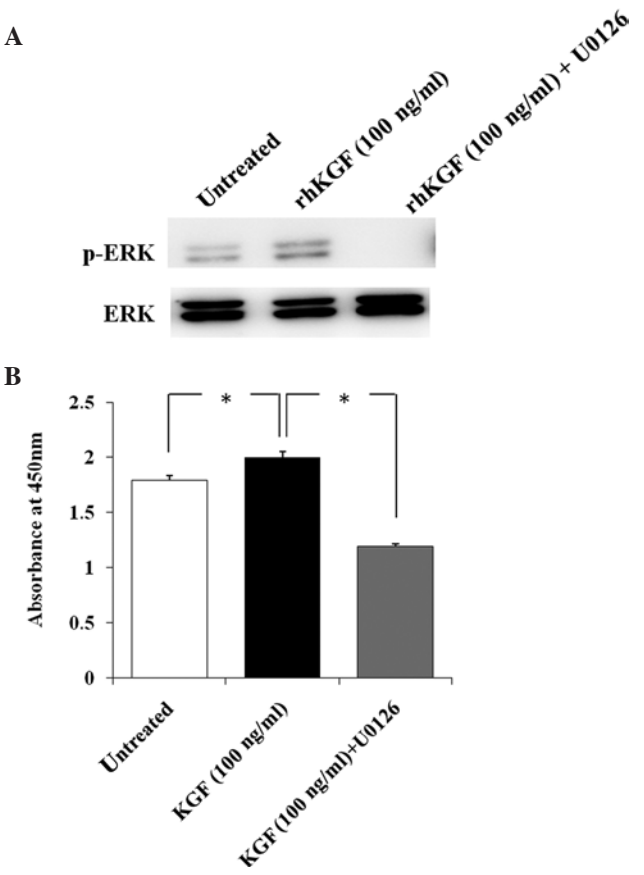

Figure 3. Effects of inhibition of the ERK signaling pathway on KGF-induced cell growth. (A) ERK phosphorylation by rhKGF was completely inhibited by pre-treatment with U0126. (B) Cell growth of rhKGF treated MIA PaCa-2 cells was significantly decreased when ERK activation was inhibited. *$p<0.05$.

100 ng/ml rhKGF (Fig. 2, third panel from top, p-p38), and JNK was not activated by the addition of rhKGF (Fig. 2, fifth panel from top, p-JNK).

Effect of inhibition of the ERK signaling pathway on KGF-induced cell growth. To determine whether ERK activation by rhKGF stimulates MIA PaCa-2 cell growth, MIA PaCa-2 cells were pre-treated with U0126 to selectively inhibit the ERK signaling pathway. ERK activation by rhKGF was completely inhibited by pre-treatment with U0126 (Fig. 3A). We then performed the cell growth assay under the same conditions and found the cell growth of rhKGF-treated MIA PaCa-2 cells to be significantly decreased after 48 h when ERK activation was inhibited (Fig. 3B, $p<0.05$).

Discussion

Exogenous KGF has been reported to induce cancer cell proliferation in several types of cancer. Exogenous KGF stimulated the growth of 5 of 35 human solid tumor cell lines, including two lung cell lines, one breast, one stomach and one colorectal cancer cell line (16,17). Following KGF treatment, ER-positive breast cancer cells exhibited a rapid increase in proliferation and motility and increased metastatic potential via the activated ERK signaling pathway (18-20). In the present study, rhKGF directly induced the cell growth of PDAC cells in a dose-dependent manner, which is consistent with other cancer cells.

To clarify the intracellular signaling pathway in rhKGF involved in PDAC cell growth, we examined the activation of three MAPK pathways. Exogenous KGF markedly activated the ERK signaling pathway in a dose-dependent manner. In contrast, p38 was only activated at high concentrations of rhKGF, while JNK was not activated even at a high concentration of rhKGF. These findings indicate that rhKGF mainly stimulates the ERK signaling pathway of the MAPK pathway, and the ERK pathway contributes to KGF-induced PDAC cell growth. We then examined the inhibitory effect of the ERK signaling pathway on KGF-induced cell growth using a specific inhibitor. Inhibition of the ERK signaling pathway clearly decreased the KGF-induced cell growth of MIA PaCa-2 cells. Therefore, the ERK signaling pathway is considered a significant pathway in KGF-induced PDAC cell growth. The cell growth rate of MIA PaCa-2 cells when the ERK signaling pathway was inhibited was lower than that of the untreated MIA PaCa-2 cells. It has been reported that MIA PaCa-2 cells produce several growth factors including FGF-2 (21), and these growth factors induced cell growth to activate the ERK signaling pathway (22,23). Thus, treatment with the ERK inhibitor may suppress the autocrine-loops of these growth factors, leading to lower growth rates compared to the untreated cells.

In conclusion, the ERK signaling pathway plays a significant role in KGF-induced cell growth in PDAC cells. These findings indicate that KGF may directly contribute to the growth of a certain type of PDAC cell, which express KGFR, to activate the ERK signaling pathway, and may be involved in the progression of pancreatic cancer.

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