Keratinocyte Growth Factor Produced by Gastric Fibroblasts Specifically Stimulates Proliferation of Cancer Cells from Scirrhous Gastric Carcinoma

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ABSTRACT

It has been previously reported (M. Yashiro et al., Jpn. J. Cancer Res., 84: 883–886, 1994) that a growth factor secreted by human gastric fibroblasts stimulated proliferation of human scirrhous gastric carcinoma cells in vitro, suggesting a similar paracrine action in the gastric submucosa. The present study established the identity of the growth factor as keratinocyte growth factor (KGF). Increase in numbers and incorporation of [3H]thymidine in scirrhous gastric carcinoma cell lines (OCUM-2M and OCUM-11) in response to culture medium from a gastric fibroblast line (NF-8 and NF-21) were duplicated by substitution of KGF and inhibited by addition of anti-KGF antibody. Effects were specific for scirrhous carcinoma cells in distinction to well-differentiated gastric carcinoma cell lines. Fibroblasts, especially gastric fibroblasts, expressed KGF mRNA, whereas gastric cancer cells did not. Conversely, scirrhous gastric cancer cells expressed more KGF receptor mRNA than well-differentiated gastric adenocarcinoma cell, whereas gastric fibroblasts did not express this mRNA. ELISA detected high concentrations of KGF in medium from gastric fibroblasts, much lower concentration in medium from other fibroblasts, and no KGF in medium from gastric cancer cells. Western analysis indicated that KGF in gastric fibroblasts lysates had a molecular weight of M₀ 19,000, within the range suggested in our previous report. Thus, gastric fibroblasts secretion of KGF is likely to underline the remarkable proliferation of scirrhous gastric cancer cells in a paracrine manner.

INTRODUCTION

Human scirrhous gastric carcinoma (diffusely infiltrating carcinoma, limitis plastica, or Borrmann type 4) is characterized by cancer cell infiltration and proliferation accompanied by extensive stromal fibrosis (1). Macrosopic findings in scirrhous gastric carcinoma indicate diffuse infiltration, whereas ulceration usually is not prominent (2). Scirrhous carcinomas account for 10% of all gastric carcinomas and carry a worse prognosis than other types of gastric carcinoma, reflecting rapid proliferation of cancer cells (3). Invading scirrhous gastric cancer cells proliferate extensively in association with fibrosis in the gastric submucosa. Mechanisms responsible for such rapid submucosal cancer cell proliferation are not clearly understood. Yashiro et al. (4) previously reported interactions between scirrhous gastric cancer cells and orthotopic fibroblasts, suggesting that proliferation of scirrhous gastric carcinoma is related to growth factor production of gastric fibroblasts. This factor had a molecular weight between M₀ 2,600 and M₀ 25,000 according to gel filtration chromatography (5). Apart from this, the growth factor had not been identified. The present investigation is the first to determine that the growth-stimulating factor from gastric fibroblasts that acts upon scirrhous gastric cancer cells is keratinocyte growth factor (KGF).

MATERIALS AND METHODS

Cell Culture and Cell Lines. The culture medium was composed of DMEM with addition of 2% heat-inactivated FCS (Life Technologies, Inc., Grand Island, NY), 100 IU/ml penicillin (ICN Biomedical, Costa Mesa, CA), 10 µg/ml streptomycin (ICN Biomedical), 2 mM glutamine (Bioproducts, Walkersville, MD), and 0.5 mM sodium pyruvate (Bioproducts). Human gastric cancer cell lines (Table 1), including OCUM-2M (poorly differentiated adenocarcinoma), OCUM-11 (poorly differentiated adenocarcinoma), MKN-28 (well-differentiated adenocarcinoma), and MKN-74 (well-differentiated adenocarcinoma) were seeded in a 100-mm dish (Falcon, Lincoln Park, NJ) and cultured in 10 ml of medium at 37°C in a humidified atmosphere containing 5% CO₂ in air. OCUM-2M and OCUM-11 were derived from scirrhous gastric carcinomas. Human fibroblast cell lines were obtained from various organs (Table 1). NF-stomach, NF-esophagus, NF-duodenum, and NF-skin were obtained from stomach, esophagus, duodenum, and the skin of a single patient, respectively. Gastric fibroblasts NF-8 and scirrhous gastric cancer cell lines OCUM-2M were obtained from the same patient. NF-21 and OCUM-11 were also obtained from another patient. WI-38, embryonic lung fibroblasts, was used as a positive control because KGF was purified from WI-38 (6).

Preparation of Serum-Free Conditioned Medium (SC-FM). SC-FM from fibroblasts was prepared as follows. Gastric fibroblasts and NF-skin (5.0 × 10⁴ cells/ml) were seeded into 100-mm plastic dishes with 10 ml of DMEM containing 2% FCS and incubated for 3 days. The number of fibroblasts in each dish was 2.5 × 10⁵ cells after 3 days of incubation. To obtain SF-CM, fibroblasts were washed twice with Dulbecco’s PBS and then incubated for 3 days in 5 ml of DMEM. CM was collected from each dish and centrifuged at 1000 × g for 5 min. The supernatant was stored as SF-CM at −20°C until use. As a control, DMEM was used instead of SF-CM.

Effect of Fibroblasts on the Growth of Scirrhous Gastric Cancer Cells. Proliferation of cancer cells or fibroblasts was determined by calculating the number of cancer cells or by measuring [3H]thymidine incorporation. After the addition of SF-CM from fibroblasts, the number of cells was calculated using a Coulter counter (Industrial D; Coulter Electronics, Luton, United Kingdom). SF-CM from fibroblasts (250 µl) was added to 750 µl of OCUM-11 cells suspension (10⁴ cells/well) with 2% FCS in each well of a 24-well plate (Falcon) and then incubated. OCUM-11 cells were counted at various time points using a Coulter counter. As a control, serum-free DMEM was used instead of SF-CM from fibroblasts. The effect of fibroblasts on DNA synthesis by scirrhous gastric cancer cells was determined by measuring [3H]thymidine incorporation. Briefly, 750 µl of scirrhous gastric cancer cell suspension (2 × 10⁴ cells/well) in DMEM with 2% FCS was added to 250 µl of SF-CM from fibroblasts in each well of a 24-well plate for incubation for 72 h at 37°C, followed by addition of 1 µCi/well [3H]thymidine (28 Ci/mmol; Amersham, Tokyo, Japan) for 12 h at 37°C. DMEM (250 µl) was used as a control. Cells were rinsed and collected in scintillation mixture, and radioactivity incorporated into the DNA was determined with a liquid scintillation counter (Aloka, Tokyo, Japan).

Effect of KGF on the Growth of Gastric Cancer Cells and Fibroblasts. To examine the effect of KGF on growth of MKN-28, MKN-74, OCUM-2M, OCUM-11, NF-8, and NF-21, KGF solution (250 µl) at various concentrations; Genzyme, Cambridge, MA) was added to 750 µl of gastric cancer cell or gastric fibroblast suspension (10⁴ cells/well) with 2% FCS in each well of a
Effect of Anti-KGF Antibodies on Growth Activity in CM from Gastric Fibroblasts. We used a neutralizing antibody for KGF, antihuman KGF antibody (Genzyme). A OCUM-2M or OCUM-11 cell suspension (10^4 cells/well in 500 μl of DMEM with 2% FCS) was inoculated into each well of a 24-well plate (Falcon). Next, 250 μl of SF-CM from NF-8 or NF-21 and antibody solution (250 μl) were added to each well for incubation. Initial solutions contained SF-CM from NF-8 or NF-21 and antibody concentrations of 0, 100, 250, or 500 ng/ml. Mouse IgG1 (Dako, Carpinteria, CA) in lieu of antibody was used as a standard for comparison. The number of OCUM-2M or OCUM-11 cells was counted after 96 h, and [3H]thymidine incorporation by OCUM-2M or OCUM-11 cells was measured after 72 h.

Reverse Transcription-PCR. Total cellular RNA was extracted from gastric cancer cells and fibroblasts with Trizol (Life Technologies, Inc.) according to the manufacturer’s protocol. Next, cDNAs were synthesized with a Moloney murine leukemia virus-reverse transcription kit (Life Technologies, Inc.) using random hexamers. The cDNAs were amplified by PCR for 30 cycles with TaqDNA polymerase (Nippon Gene, Tokyo, Japan) on a thermal cycler. The following KGF primers were used: sense, 5’-ACATGGGAAGGAGGATATAAGAG-3’ and antisense, 5’-TTCACCTTTCCACACCCCTGATTCG-3’. PCR conditions were as follows: predenaturation, 94°C for 5 min; denaturation, 94°C for 30 s; annealing, 58°C for 30 s; extension, 72°C for 1 min; and final incubation, 72°C for 10 min. PCR products for KGF were 175 bp in length. The following KGF receptor (KGFR) primers were used: sense, 5’-CTTACGCCGGCACTCCTCAA-3’ and antisense, 5’-ATCCCTACTCAGCTGCTG-3’. PCR conditions were as follows: predenaturation, 94°C for 5 min; denaturation, 94°C for 30 s; annealing, 61°C for 30 s; extension, 72°C for 1 min; and final incubation, 72°C for 10 min. PCR products for KGFR were 255 bp in length; these products were then applied to 2% agarose gel and electrophoresed. As an internal control, reverse transcription-PCR for glyceraldehyde-3-phosphate dehydrogenase was performed: sense, 5’-ACCTGACCTGCGCGGTCTGAA-3’ and antisense, 5’-TCCACACCCTGTGCTGTA-3’.

ELISA. KGF in conditioned medium from NF-8, NF-21, NF-stomach, NF-esophagus, NF-duodenum, and NF-skin were obtained from a same patient. The conditioned medium of each cell line was tested by ELISA. A single SF-CM from NF-21 cells significantly increased the number of OCUM-11 cells by 32% of cell numbers in the control group after 72 h and by 29% after 96 h (Fig. 1A). The SF-CM from NF-21 cells significantly stimulated DNA synthesis of OCUM-11 cells by 21% of synthesis in the control group (Fig. 1B). In contrast, SF-CM from NF-skin cells did not affect the growth of OCUM-11 cells.

Effect of KGF on the Growth of MKN-28, MKN-74, OCUM-2M, and OCUM-11. To investigate relationships between cell types and growth effects of KGF, we compared the effect of KGF on growth of scirrhous gastric cancer cell lines (OCUM-2M and OCUM-11) with that on growth of well-differentiated adenocarcinoma cell lines (MKN-28 and MKN-74) and gastric fibroblasts (NF-8 and NF-21). Although KGF did not affect growth of MKN-28 or MKN-74 cells (Fig. 2), 10 ng/ml KGF significantly increased the number of OCUM-2M cells and OCUM-11 cells by 19% and 24%, respectively, after 96 h (Fig. 2). KGF at 1 ng/ml also significantly increased the number of OCUM-2M cells by 16%. KGF did not affect the growth of fibroblasts and NF-8 and NF-21 cells (data not shown).

Effect of Anti-KGF Antibodies on Growth-Stimulating Activity of CM from Gastric Fibroblasts. To examine the relationship between the growth activity of SF-CM from gastric fibroblasts and KGF, we tested whether neutralizing antibodies against KGF could neutralize the growth-stimulating activity of SF-CM. The stimulating effect of SF-CM from gastric fibroblasts on the growth of OCUM-2M cells or OCUM-11 cells (Fig. 3, A and B) was significantly inhibited by addition of anti-KGF antibody in a dose-dependent manner, compared with a standard group with IgG1 instead of antibody. Bioactivity of the neutralizing antibody against KGF had been characterized in a preliminary experiment (data not shown).

Expression of KGF and KGFR mRNA by Gastric Cancer Cells and Fibroblasts. We investigated degree of expression of KGF and KGFR mRNA in gastric cancer cells and fibroblasts. KGF mRNA of cancer cells was measured in SF-CM from NF-21 or NF-8 cells on [3H]thymidine uptake by cancer cells. SF-CM from NF-21 cells significantly increased the number of OCUM-11 cells by 32% after 72 h and by 29% after 96 h in culture compared with the control (D), whereas SF-CM from NF-skin cells (C) did not affect the growth of OCUM-11 cells. A single asterisk denotes a statistically significant difference from control values (P < 0.05). SF-DMEM was used as the control. Data are presented as the mean and SD (bars) of three independent experiments. B, effect of SF-CM from NF-21 or NF-skin cells on [3H]thymidine uptake by cancer cells. SF-CM from NF-21 cells significantly stimulated the DNA synthesis in OCUM-11 cells by 21% compared with the control, whereas SF-CM from NF-skin cells did not affect growth of OCUM-11 cells. A single asterisk denotes a statistically significant difference from the control values (P < 0.05). SF-DMEM was used as the control. Data are presented as the mean and SD (bars) of three independent experiments.
175 bp was found to be expressed in gastric fibroblasts (NF-8 and NF-21). KGF mRNA was expressed in gastric fibroblasts, esophageal fibroblasts, duodenal fibroblasts, and lung fibroblasts but not in gastric cancer cells and skin fibroblasts (Fig. 4). KGF mRNA of 255 bp was expressed in gastric cancer cells and was not expressed in fibroblasts (Fig. 5).

**KGF Amounts in CM.** We measured concentrations of KGF in CM from gastric cancer cells and fibroblasts by ELISA. KGF concentrations in CM from NF-8, NF-21, NF-stomach, NF-esophagus, and NF-duodenum were 840, 834, 682, 184, and 31 pg/ml, respectively. In contrast, KGF was undetectable in CM from NF-skin, MKN-28, MKN-74, OCUM-2M, and OCUM-11 (Table 2).

**Molecular Weight of KGF.** We investigated whether KGF produced by gastric fibroblasts had a molecular weights between Mr 2600 and Mr 25,000. Western analysis indicated that gastric fibroblasts, esophageal fibroblasts, duodenal fibroblasts, and lung fibroblasts produced a Mr 19,000 KGF (Fig. 4).

**DISCUSSION**

Yashiro et al. (5) previously reported that a protein with a molecular weight from Mr 2,600 to Mr 25,000 produced by gastric fibroblasts had a growth-promoting effect on scirrhous gastric carcinoma cells. Fibroblasts have been reported to produce various growth factors (6–11). We previously examined whether any defined growth factors, including epidermal growth factor, vascular endothelial growth factor, transforming growth factor α, basic fibroblasts growth factor, insulin-like growth factor I, platelet-derived growth factor, hepatocyte growth factor, and transforming growth factor β, was the active factor in the CM from gastric fibroblasts. None of these growth factors was associated with a protein of molecular weight from Mr 2,600 to Mr 25,000 (5). In the present study, we concluded that the growth-stimulating factor from gastric fibroblasts that affected scirrhous gastric cancer cells is KGF. We previously reported that KGF has an especially high growth-stimulating activity in scirrhous gastric...
cancer cells. To investigate whether histological type of gastric cancer cells influenced KGF effects, we compared the effect of KGF on the growth of scirrhous gastric cancer cell lines (OCUM-2M and OCUM-11) with growth effects in well-differentiated adenocarcinoma cell lines (MKN-28 and MKN-74). KGF significantly stimulated the growth of scirrhous gastric cancer cells but not that of well-differentiated adenocarcinoma cells. These results suggest that the growth effect of KGF depended upon the histological type of gastric cancer cells.

To determine whether the growth factor in SF-CM from gastric fibroblasts is KGF, we tested whether neutralizing antibodies against KGF affected the growth-stimulating activity of gastric fibroblast CM. Activity was dose dependently inhibited by neutralizing antibodies against KGF. These *in vitro* findings suggested that KGF secreted by gastric fibroblasts *in vivo* stimulate growth of scirrhous gastric cancer cells in a paracrine manner. KGF mRNA was expressed in gastric fibroblasts but not in gastric cancer cells and skin fibroblasts. Western analysis indicated that orthotopic fibroblasts produced a Mr 19000 KGF, whereas KGF was undetectable in gastric cancer cells. These results agree with our previous findings that growth of scirrhous gastric cancer cells was significantly enhanced by SF-CM from orthotopic fibroblasts, but not by SF-CM from ectopic fibroblasts, and that the molecular weight of the growth factor was between Mr 2600 and Mr 25,000 (5). We therefore concluded that the growth factor secreted by gastric fibroblasts to stimulate scirrhous gastric cancer cell growth is KGF.

KGF, a member of the fibroblast growth factor (FGF) family, also known as FGF-7 (6, 12), originally was isolated from human embryonic lung fibroblasts (6, 13) and is produced by mesenchymal cells in various tissues (14–17). KGF exerts its effect in a paracrine manner limited to epithelial cells, whereas other FGF family members also stimulate growth of cultured endothelial cells and fibroblasts (6, 13, 18). Several types of FGF receptors have been reported, FGF receptor 2 or KGFR is identical to the K-sam-II gene product. The K-sam-II gene first was amplified and identified in an extract from the human gastric cancer cell line KATO-III (19). K-Sam-II has been reported to be preferentially expressed in scirrhous gastric cancer (20). The scirrhous gastric cancer cell lines OCUM-2M and OCUM-11 also strongly expressed K-sam-II (21). In our present study, abundant KGFR mRNA was amplified from scirrhous gastric cancer cells (OCUM-2M and OCUM-11), whereas the ligands KGF was produced by gastric fibroblasts. These findings suggested that KGF secreted by gastric fibroblasts is important in progression of scirrhous gastric cancer. Liver metastasis is one of frequent types of metastases in gastric cancer. It has been reported that KGF mRNA was detectable in gastrointestinal tract but not in liver (15). Therefore, the expression pattern of KGF mRNA might not be useful to predict preferential sites of metastasis.

Yashiro *et al.* (4) previously reported that the tumorigenicity of coinoculation of scirrhous gastric cancer cells with gastric fibroblasts in nude mice was increased considerably compared with that of inoculation of scirrhous gastric cancer cells alone. The tumor size observed at 4 weeks after the coinoculation of gastric cancer cells and gastric fibroblasts was ~5 times larger than that of gastric cancer cells alone (22). In addition, it has been reported that the proliferation of fibroblasts was increased by SF-CM from scirrhous gastric cancer cells *in vitro* (23). In this study, CM from gastric fibroblasts increases the final density of gastric cancer cells in a growth assay by 30% compared with DMEM control *in vitro*. These findings suggested that the growth-promoting factors from gastric cancer cells and fibroblasts might mutually increase each other’s proliferation *in vivo* and *in vitro*, which resulted in a rapid development of scirrhous gastric carcinoma. The growth difference of CM from fibroblasts was approximately only a 30% increase of cell numbers *in vitro*. However, this increase indicates only the growth effect from fibroblasts on cancer cells but not from cancer cells on fibroblasts. *In vivo*, growth interaction continuously exists between cancer cells and fibroblasts. The continuous intercellular interaction may mutually develop much more growth effect than the 30% increase. Therefore, a few increases of growth difference *in vitro* might result in a rapid development of scirrhous gastric carcinoma *in vivo* by intercellular interaction.

In conclusion, the growth of scirrhous gastric cancer cells was closely associated with KGF produced from gastric fibroblasts. This is the first report to identify the growth factor from gastric fibroblasts that stimulated progression of scirrhous gastric carcinoma as KGF.

### REFERENCES


### Table 2. KGF production from cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Keratinocyte growth factor (pg/ml)</th>
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<tbody>
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<td>NF-8</td>
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<tr>
<td>NF-21</td>
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<td>182</td>
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<tr>
<td>NF-duodenum</td>
<td>&lt;31.2</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>&lt;31.2</td>
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<tr>
<td>MKN-74</td>
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* The minimum detectable concentration was 31.2 pg/ml in this assay.

**Fig. 5. Keratinocyte growth factor receptor (KGFR) mRNA expression.** KGFR mRNA of 255 bp was expressed in gastric cancer cells, and was not expressed in gastric fibroblasts. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.


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