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Transforming Growth Factor-β and Hepatocyte Growth Factor Produced by Gastric Fibroblasts Stimulate the Invasiveness of Scirrhous Gastric Cancer Cells

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Scirrhous gastric carcinoma is characterized by cancer cells that infiltrate rapidly in the stroma with extensive growth of fibroblasts. In the present study, we examined the effect of gastric fibroblasts on the invasiveness of a scirrhous gastric cancer cell line, OCUM-2D, using an invasion assay. Gastric fibroblast-derived conditioned medium (CM) significantly stimulated the invasiveness of OCUM-2D cells, as did transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF). The stimulating activity of gastric fibroblast-derived CM was inhibited significantly by anti-TGF-β neutralizing antibody or anti-HGF neutralizing antibody. TGF-β and HGF were detected in the gastric fibroblast-derived CM, and TGF-β receptor and C-met (HGF receptor) were expressed on OCUM-2D cells. Thus, TGF-β and HGF produced by gastric fibroblasts appear to affect the invasiveness of scirrhous gastric cancer cells. TGF-β was also detected in the conditioned medium derived from OCUM-2D cells, though HGF was not. TGF-β appears to affect the invasiveness of OCUM-2D cells in both paracrine and autocrine fashions.

Key words: Scirrhous gastric carcinoma — Fibroblast — Invasion — Transforming growth factor-β — Hepatocyte growth factor

Patients with scirrhous gastric carcinoma have a poor prognosis because of the high frequency of peritoneal metastasis.[1-3] Early diagnosis of scirrhous gastric carcinoma is very difficult and infrequent. When scirrhous gastric cancer cells in the mucosa invade the submucosa of the stomach, cancer cells infiltrate diffusely and rapidly through the gastric wall with extensive growth of fibroblasts. Scirrhous gastric carcinoma (Borrman's type 4 carcinoma) is characterized by histological findings of cancer cells which diffusely infiltrate and cause massive fibrosis in the stroma.[4] Recently there have been several studies of the effect of the microenvironment on the invasiveness of cancer cells, covering gallbladder cancer, renal cell cancer, colon cancer and melanoma.[5-11] It is thought that organ-specific fibroblasts play an important role in cancer proliferation and invasion.[2,4,6,11] Thus, the invasiveness of scirrhous gastric cancer cells might be influenced by gastric fibroblasts. However, there has been no report mentioning the effect of gastric fibroblasts on the invasiveness of scirrhous gastric cancer cells. Knowledge of the mechanism of this rapid infiltration of cancer cells should allow the development of measures to inhibit it, improving the prognosis of patients with scirrhous gastric carcinoma. In this study, we examined the effect of the factors produced by gastric fibroblasts on the invasiveness of scirrhous gastric cancer cells.

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MATERIALS AND METHODS

Cell culture OCUM-2D, a human gastric cancer cell line, was derived from the pleural effusion of a patient with scirrhous gastric carcinoma.[3] NF-8, a human fibroblast cell line, was derived from the stomach of the same patient. NF-8 was established in our laboratory as reported previously.[12] Fibroblast origin was verified by immunostaining with monoclonal antibodies against human fibroblasts (Dako, Glostrup, Denmark). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Bioproducts, Walkersville, MD) with 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY), 100 IU/ml of penicillin (ICN Biomedicals, Costa Mesa, CA), 100 μg/ml of streptomycin (ICN Biomedicals), 2 m*M* glutamine (Bioproducts), and 0.5 m*M* sodium pyruvate (Bioproducts).

Preparation of gastric fibroblast- and cancer cell-derived conditioned medium Gastric fibroblasts, NF-8, were seeded into a 100 mm plastic dish (Falcon, Lincoln Park, NJ) and incubated in DMEM with 10% FCS to a semi-confluent monolayer state. After removal of the supernatant, cells were washed twice in phosphate-buffered saline (PBS; Bioproducts). Then, the cells were incubated in 3 ml of DMEM with 10% FCS for 3 days or in 3 ml of DMEM without FCS for 2 days. The medium was collected as conditioned medium from NF-8 (CM NF-8) or serum-free CM from NF-8 (SF-CM NF-8), respectively.

The medium was centrifuged at 1,000*g* for 5 min, filtered through a microfilter (pore size, 0.45 μm; Kurabo, Osaka) and stored at −20°C until use. The fibroblasts, NF-8, were used before the 30th passage in culture. Serum-free CM from OCUM-2D (SF-CM OCUM-2D) was collected by the same method.

Invasion assay *In vitro* invasiveness was measured by the method of Albini *et al.* with some modifications.[13] We used Chemotaxicell chambers (Kubota, Osaka) with a 12 μm membrane filter, and the upper surface of each filter was coated with 5 μg of Matrigel (reconstituted basement membrane: Collaborative Research, Lexington, MA) in cold DMEM to form a matrix barrier. The chamber (upper compartment) was placed in a 24-well culture plate (lower compartment). OCUM-2D cells were resuspended to a final concentration of 1×10^5 cells/ml in DMEM with 10% FCS. One hundred microliter of cancer cell suspension and 900 μl of DMEM with 10% FCS were added to the upper and lower compartments, respectively, and the plate was incubated for 72 h at 37°C. After incubation, cancer cells on the upper surface of the membrane were removed by wiping with cotton swabs. The filter was fixed with methanol and stained with hematoxylin. Cancer cells that invaded through a filter coated with Matrigel to the lower surface of the membrane were counted manually under a microscope at ×200 magnification. For each group, the culture was performed in triplicate.

Effect of gastric fibroblast-derived CM: CM NF-8 were added at a concentration of 25%, 50%, 75%, and 100% in DMEM with 10% FCS in the lower compartment. DMEM with 10% FCS was used as the control.

Effect of defined cytokines: The effects of various defined cytokines, including transforming growth factor-β (TGF-β; King Brewing, Kakogawa), hepatocyte growth factor (HGF; Otsuka, Tokushima), epithelial growth factor (EGF; Gibco), basic fibroblast growth factor (bFGF; Austral Biologicals, San Ramon, CA), vascular endothelial growth factor (VEGF; Genzyme, Cambridge, MA), platelet-derived growth factor (PDGF; Austral Biologicals, San Ramon, CA), transforming growth factor-α (TGF-α; Becton Dickinson Lab., Bedford, MA) and interleukin-1β (IL-1β; Genzyme), on the invasiveness of OCUM-2D cells were examined. These factors have been shown to be produced by fibroblasts.[10] The factors were reconstituted in PBS with 0.1% bovine serum albumin (BSA) at the concentration of 0.01–10 μg/ml. A 10 μl aliquot of these solutions was added to 890 μl of DMEM with 10% FCS in the lower chamber. The final concentrations of the factors were 0.1–100 ng/ml. PBS with 0.1% BSA was used as the control.

Effect of neutralizing antibodies: The effect of the anti-TGF-β neutralizing antibody and anti-HGF neutralizing antibody on the invasiveness of OCUM-2D cells was examined. Anti-TGF-β antibody (Genzyme), anti-HGF antibody (Sigma, St. Louis, MO) and mouse IgG1 standard (TAGO, Burlingame, CA) were used. Each antibody was reconstituted in PBS with 0.1% BSA at a concentration of 0.1–10 mg/ml. These solutions was added to DMEM with 10% FCS with or without 50% CM NF-8. The final concentration of each antibody was 1–100 μg/ml.

Proliferation assay of scirrhous gastric cancer cells OCUM-2D cells (1.0×10^4) were seeded on a 12-well plastic plate (Falcon) and incubated with each factor in 1 ml of DMEM containing 10% FCS for 36 h and 72 h. Then, the cells were dispersed by trypsin/EDTA (Gibco) treatment and counted with a Coulter counter. The factors we used were 50% CM NF-8, 10 ng/ml of TGF-β and 100 ng/ml of HGF. The concentration of each factor that showed the maximum effect on invasion assay was employed. DMEM with 10% FCS was used as the control.

Western blot analysis Production of TGF-β and HGF was studied by means of western blot analysis. Concentrated serum-free medium was used. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[14] A 10 μl aliquot of SF-CM was run on 10% SDS-polyacrylamide gel under non-reducing conditions. Proteins in the gel were electrophoretically transferred onto Immobilon-P membrane (Millipore, Bedford, MA). The antigen on the membrane was probed with anti-TGF-β antibody or anti-HGF antibody. After washing the membrane with PBS, the antigen-antibody complex was probed with peroxidase-conjugated anti-mouse IgG (Medical and Biological Labo., Nagoya). Then, to visualize the blots, the membrane was incubated in 3-amino-9-ethylcarbazole buffer.

Enzyme-linked immunosorbent assay (ELISA) The quantitation of TGF-β and HGF in the conditioned medium was performed by using a TGF-β ELISA Kit (King Brewing) and an HGF ELISA Kit (Otsuka Pharmaceutical, Tokyo), respectively.

Flow cytometry To estimate the expression of TGF-β receptor of the OCUM-2D cells, a flow cytometric analysis kit (R&D Systems, Minneapolis, MN) was used. OCUM-2D cells were prepared as a single cell suspension, and then washed twice with PBS to remove residual TGF-β present in the culture medium. Cells were resuspended in FACS buffer (PBS with 1% BSA with 0.1% sodium azide) at a final concentration of 1×10^6 cells/ml to block nonspecific binding. Biotinylated rh TGF-β1 (1 μg/ml) was added to the OCUM-2D cell suspension and the cells were incubated for 60 min at 4°C. After the incubation, avidin fluorescence isothiocyanate (FITC) was added and the cells were washed twice with PBS and analyzed using a flow cytometer. Biotinylated soybean

trypsin inhibitor was used as a negative control. The expression of C-met of the OCUM-2D cells was also estimated by using flow cytometric analysis as follows. The OCUM-2D cells were prepared as a single cell suspension, and then washed twice with PBS. Cells were resuspended in FACS buffer at a final concentration of 1×10^6 cells/ml. Anti-C-met antibody (Santa Cruz Biotechnology, CA) was added to the OCUM-2D cell suspension and incubated for 60 min at 4°C. After incubation, FITC-labeled anti-rabbit IgG antibody (Organon Teknika, West Chester, PA) was added. Cells were washed twice and analyzed using a flow cytometer. Standard rabbit IgG (Organon Teknika) was used as a negative control.

Statistical analysis Data were analyzed statistically using Student's t test. A P-value less than 0.05 was considered significant.

RESULTS

Effect of gastric fibroblasts on the invasiveness Fig. 1 shows the effect of CM NF-8 on the invasiveness of OCUM-2D cells. Addition of 50% and 75% CM NF-8 significantly increased the invasiveness of OCUM-2D cells. The maximum stimulation of the invasiveness was observed upon addition of 50% CM NF-8. The effect of addition of 100% CM NF-8 did not cause any significant difference compared to the control. These results suggested that factors produced from NF-8 stimulated the invasiveness of OCUM-2D cells.

Effect of defined cytokines on the invasiveness The effects of various cytokines which are produced by fibroblasts on the invasiveness of OCUM-2D cells were studied (Fig. 2). The invasiveness of OCUM-2D cells was increased by the addition of TGF-β or HGF. The addition of EGF, bFGF, VEGF, PDGF, TGF-α or IL-1β had no effect on the invasiveness. The effect of TGF-β reached a maximum at a concentration of 10 ng/ml, and a concentration of 100 ng/ml did not produce a greater effect. HGF increased the invasion ability in a dose-dependent manner and the maximum stimulatory effect of HGF was observed at a concentration of 100 ng/ml. Less than 10 ng/ml of HGF had no significant effect on the invasiveness. PBS with 0.1% BSA was used as the control.

Effect of neutralizing antibodies on the invasiveness The stimulating effect of TGF-β or HGF on the invasiveness of OCUM-2D cells was significantly inhibited by addi-

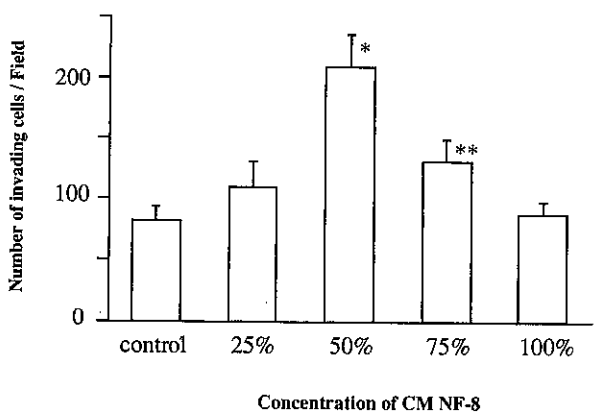

Fig. 1. Effect of gastric fibroblast-derived CM on invasiveness. The invasiveness of OCUM-2D cells was evaluated by invasion assay. OCUM-2D cells were added to the upper compartment and each factor was added to the lower compartment of the chamber. The chamber was incubated for 72 h at 37°C. The cells that invaded through the Matrigel-coated 12 μm membrane were stained with hematoxylin and counted manually under microscope. The invasiveness of OCUM-2D cells was increased significantly following the addition of 50% and 75% CM NF-8. DMEM with 10% FCS was used as the control. Data are presented as the mean of 4 samples and the error bar shows the SD. The significance of any difference was determined by using Student's t test. $*P<0.01$, $**P<0.05$ versus the control.

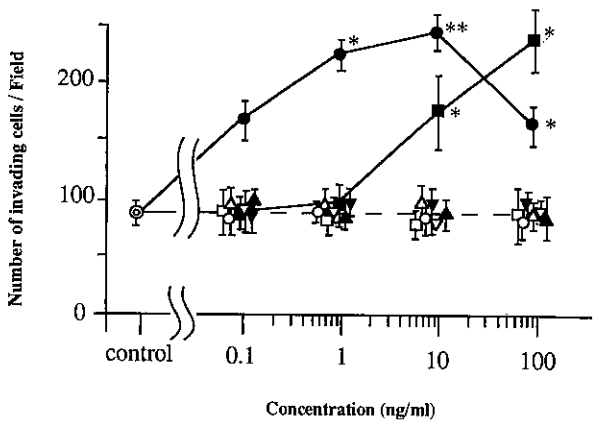

Fig. 2. Effect of defined cytokines on invasiveness. The invasiveness of OCUM-2D cells was increased significantly by TGF-β and HGF. The addition of EGF, bFGF, VEGF, PDGF, TGF-α and IL-1β had no effect. The maximum stimulative effect of TGF-β was observed at the concentration of 10 ng/ml. HGF increased the invasion ability in a dose-dependent manner and the maximum stimulative effect of HGF was observed at the concentration of 100 ng/ml. PBS with 0.1% BSA was used as the control. Data are presented as the mean of 4 samples and the error bar shows the SD. The significance of any difference was determined using Student's t test. $*P<0.05$, $**P<0.01$ versus the control. ◎ control, ● TGF-β, ■ HGF, □ EGF, ○ bFGF, △ VEGF, ▽ PDGF, ▼ TGF-α, ▲ IL-1β.

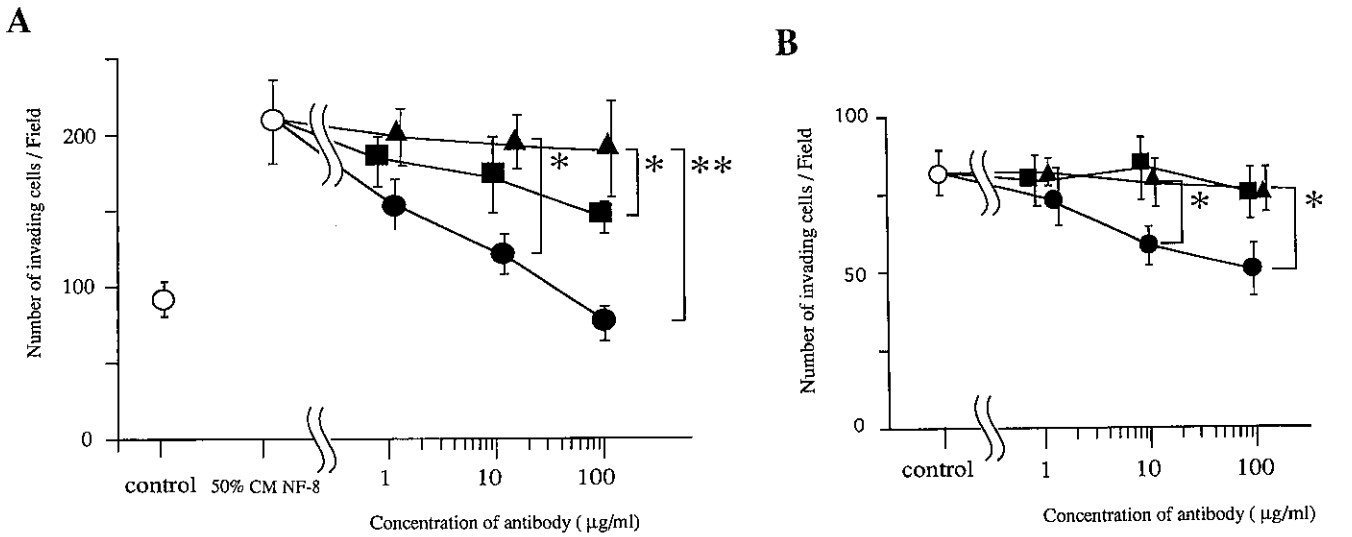

Fig. 3. Effects of neutralizing antibody on invasiveness. A shows the effect of anti-TGF-β antibody (●), anti-HGF antibody (■) and standard IgG (▲) on the invasiveness of OCUM-2D cells with 50% CM NF-8 and B shows that without 50% CM NF-8. DMEM with 10% FCS was used as the control in both cases. Anti-TGF-β antibody and anti-HGF antibody partly inhibited the stimulating effect of 50% CM NF-8 on the invasiveness of OCUM-2D cells in a dose-dependent manner. The invasiveness of OCUM-2D cells without addition of CM NF-8 was partly inhibited by anti-TGF-β antibody, but was unaffected by anti-HGF antibody. Data are presented as the mean of 4 samples and the error bar shows the SD. The significance of any difference was determined using Student's t test. $*P<0.05$, $**P<0.01$ versus 50% CM NF-8 with standard IgG in A and versus the control with standard IgG in B.

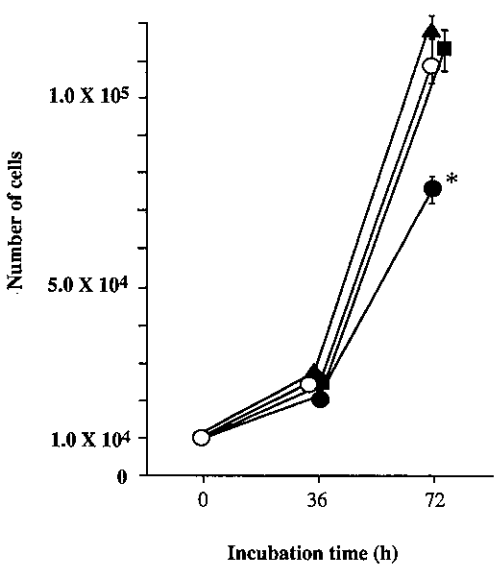

Fig. 4. Effect of various factors on proliferation. OCUM-2D cells (1.0×10^4) were seeded and incubated with each factor in 1 ml of DMEM containing 10% FCS for 36 h and 72 h. Then, cells were counted with a Coulter counter. The addition of 50% CM NF-8 or that of 100 ng/ml of HGF did not significantly affect the proliferation of OCUM-2D cells after 36 h or 72 h incubation. The addition of 10 ng/ml of TGF-β significantly inhibited the growth of OCUM-2D cells after 72 h incubation. DMEM with 10% FCS was used as the control. Data are presented as the means of four samples and the error bar shows the SD. The significance of any difference was determined using Student's t test. $*P<0.01$, versus the control. ○ control, ▲ CM-NF-8, ● TGF-β, ■ HGF.

tion of anti-TGF-β antibody or anti-HGF antibody, respectively (data not shown). The stimulating effect of 50% CM NF-8 on the invasiveness of OCUM-2D cells was also significantly inhibited by addition of anti-TGF-β antibody or anti-HGF antibody in a dose-dependent manner (Fig. 3A). In the range of concentration used in this study, the effect of anti-TGF-β antibody was greater than that of anti-HGF antibody. The invasiveness of

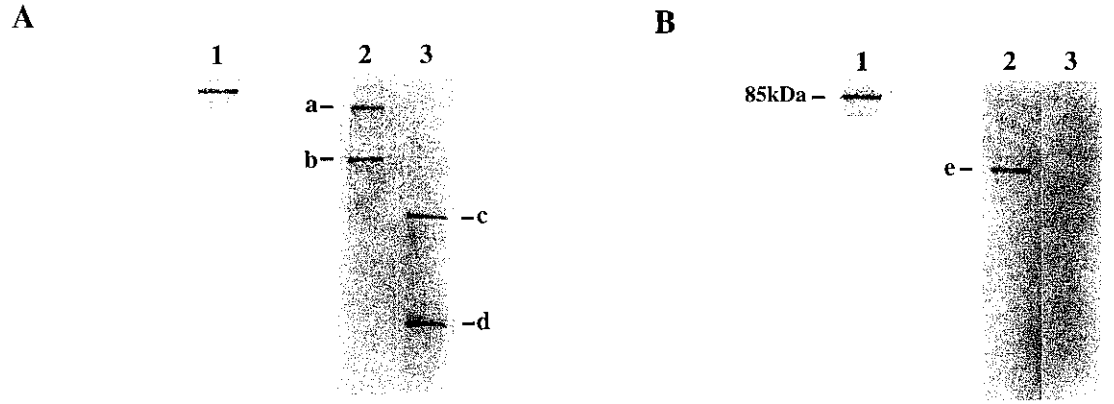

Fig. 5. The production of TGF-β and HGF. TGF-β and HGF were detected in the conditioned medium by western blot analysis. Western blot analysis was performed as described in "Materials and Methods." A-1 and B-1 show positive control TGF-β and HGF, respectively. TGF-β was detected at molecular weights of 105 kDa (a) and 52.5 kDa (b) in SF-CM NF-8 under non-reducing conditions (A-2) and at molecular weights of 25 kDa (c) and 12.5 kDa (d) in SF-CM OCUM-2D under non-reducing conditions (A-3). HGF was detected at a molecular weight of 85 kDa (e) under non-reducing conditions in SF-CM NF-8 (B-2), but was not detected in SF-CM OCUM-2D (B-3).

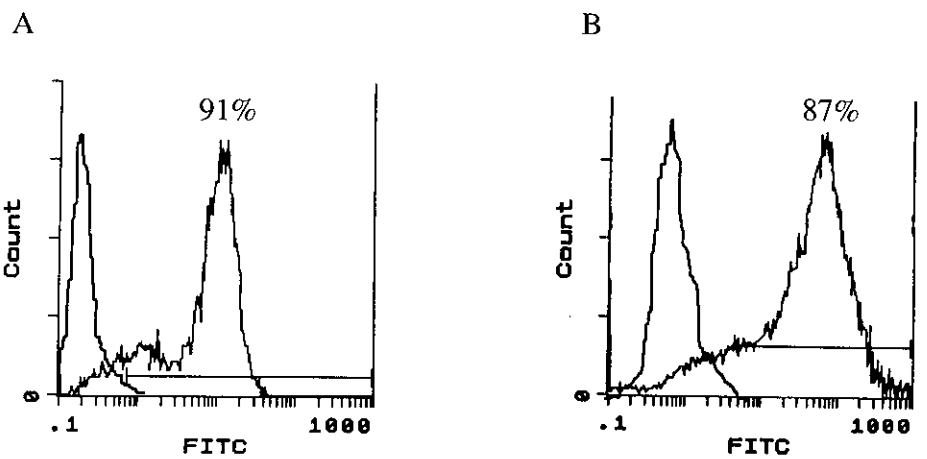

Fig. 6. Expression of TGF-β receptor and C-met on OCUM-2D cells. The expression of the receptors of TGF-β and HGF was detected by flow cytometric analysis as described in "Materials and Methods." The expression of TGF-β receptor was detected in 91% of OCUM-2D cells (A). Biotinylated soybean trypsin inhibitor was used as a negative control. The expression of C-met was detected in 87% of OCUM-2D cells (B). Standard mouse IgG was used as a negative control.

OCUM-2D cells without addition of CM NF-8 was significantly inhibited by anti-TGF-β antibody, but not by anti-HGF antibody (Fig. 3B).

Effect of factors on the proliferation of scirrhous gastric cancer cells The effect of the factors that stimulated the invasiveness of OCUM-2D cells on the cell proliferation were studied (Fig. 4). Addition of 50% CM NF-8 and 100 ng/ml of HGF had no significant effect after 36 h and 72 h incubation. However addition of 10 ng/ml of TGF-β significantly inhibited the proliferation of OCUM-2D cells. These results suggest that the factors which stimulate invasiveness of OCUM-2D cells did not have a proliferation-stimulating activity, at least under these conditions.

Production of TGF-β and HGF Production of TGF-β and HGF in the conditioned medium was detected by means of western blot analysis and ELISA. Fig. 5, A-1 and B-1 showed positive control TGF-β and HGF-β, respectively. TGF-β was detected at molecular weights of 105 kDa and 52.5 kDa in SF-CM NF-8 (Fig. 5A-2) and

at molecular weights of 25 kDa and 12.5 kDa in SF-CM OCUM-2D under non-reducing conditions (Fig. 5A-3). HGF was detected at a molecular weight of 85 kDa under non-reducing conditions in SF-CM NF-8 (Fig. 5B-2), but not in SF-CM OCUM-2D (Fig. 5B-3). The concentrations of TGF-β in CM NF-8 and CM OCUM-2D were 4.2 ng/ml and 1.3 ng/ml, respectively. That of HGF in CM NF-8 was 16.6 ng/ml, while HGF was undetectable in CM OCUM-2D. The minimum detectable concentration was 0.3 ng/ml in this assay.

Expression of TGF-β receptor and C-met on OCUM-2D cells The expression of TGF-β receptor and C-met on OCUM-2D cells was examined by flow cytometric analysis. The expression of TGF-β receptor and C-met was detected in 91% and 87% of OCUM-2D cells, respectively (Fig. 6, A and B). OCUM-2D cells had receptors for both TGF-β and HGF.

DISCUSSION

In this study, gastric fibroblast-derived conditioned medium increased the invasiveness of scirrhous gastric cancer cells. Fibroblasts have been reported to produce various cytokines, such as TGF-β, HGF, EGF, bFGF, VEGF, PDGF, TGF-α and IL-1β.[6] Our fibroblast cell line also produced TGF-β and HGF, and recombinant TGF-β and HGF significantly stimulated the invasiveness of OCUM-2D cells. The stimulating activity of CM NF-8 was significantly inhibited by anti-TGF-β or anti-HGF neutralizing antibody. In addition, recombinant TGF-β and HGF did not stimulate the proliferation of OCUM-2D cells at the concentration that showed the maximum effect on invasion assay. These findings suggest that TGF-β and HGF produced by gastric fibroblasts may stimulate the invasiveness of scirrhous gastric cancer cells. With regard to the concentration of CM NF-8, the degree of invasiveness of the OCUM-2D cells seen in 75% and 100% CM NF-8 was lower than that in 50% CM NF-8. The reason is not clear. However, CM NF-8 was the spent medium in which NF-8 cells had been incubated for three days, so some factors necessary for invasion of OCUM-2D cells might have been depleted.

TGF-β has been reported to increase the invasiveness of various cancer cells, including glioma, melanoma and breast cancer.[15–18] Immunohistochemical studies have shown that TGF-β is expressed more intensely in scirrhous gastric cancer than non-scirrhous gastric cancer.[19] Our previous study indicated that TGF-β changed the morphology of OCUM-2D cells from round to spindle-shaped,[3] and the morphologic change to spindle shape was reported to increase the invasiveness.[17, 20] It has been reported that TGF-β modulates the synthesis of proteolytic enzymes such as gelatinase A, gelatinase B and urokinase-type plasminogen activator (u-PA), which are associated with cancer invasion.[4, 17, 21, 22] Thus, TGF-β produced from gastric fibroblasts and cancer cells themselves may affect the invasiveness of scirrhous gastric cancer cells by inducing morphologic change to a spindle shape and by stimulating protease production.

HGF stimulates the invasiveness of many kinds of transformed cells or non-transformed cells.[1, 2, 23–27] It has been reported that the c-*met* gene, which encodes C-met as the HGF receptor[28, 29] was amplified more intensely in scirrhous gastric cancer than in non-scirrhous gastric cancer.[30, 31] However, there has been no report dealing with the effect of HGF on the invasiveness of scirrhous gastric cancer cells. Flow cytometric analysis indicated that OCUM-2D cells express C-met on the cell surface. Exogenous recombinant HGF stimulated the invasiveness of OCUM-2D cells, and HGF was produced by NF-8. These results suggest that HGF produced by gastric fibroblasts also stimulated the invasiveness of OCUM-2D cells.

TGF-β was detected in both CM NF-8 and CM OCUM-2D. Anti-TGF-β neutralizing antibody significantly inhibited the invasiveness of OCUM-2D cells with or without addition of CM NF-8. Thus, TGF-β may stimulate the invasiveness of scirrhous gastric cancer cells in both paracrine and autocrine fashions. HGF was not detected in CM OCUM-2D, and anti-HGF neutralizing antibody did not affect the invasiveness of OCUM-2D cells without addition of CM NF-8. These results suggest that HGF influences the invasiveness of OCUM-2D cells in a paracrine fashion, but not in an autocrine fashion. The highly invasive character of scirrhous gastric cancer cells seems to be caused by factors produced by gastric fibroblasts in a paracrine fashion and by factors produced by the cancer cells in an autocrine fashion.

TGF-β was detected at molecular weights of 105 kDa and 52.5 kDa in SF-CM NF-8 and was detected at molecular weights of 25 kDa and 12.5 kDa in SF-CM OCUM-2D under non-reducing conditions. TGF-β was detected as a monomer and a dimer in latent form in the CM from fibroblasts and as a monomer and a dimer in active form in the CM from scirrhous gastric cancer cells. Various types of cells secrete TGF-β in the latent form, and latent TGF-β is activated by proteases such as plasmin and cathepsin.[32–35] OCUM-2D cells produce u-PA, and it was reported that u-PA converts latent TGF-β to active TGF-β.[35] Thus, latent TGF-β from gastric fibroblasts and scirrhous gastric cancer cells may be activated by u-PA from scirrhous gastric cancer cells.

In conclusion, TGF-β and HGF produced by gastric fibroblasts appear to affect the invasiveness of scirrhous gastric cancer cells in a paracrine fashion. TGF-β produced by gastric cancer cells may also affect the invasiveness in an autocrine fashion.

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REFERENCES

1) Kiyasu, Y., Kaneshima, S. and Koga, S. Morphogenesis of peritoneal metastasis in human gastric cancer. *Cancer Res.*, **41**, 1236–1239 (1981).
2) Yashiro, M., Chung, Y. S., Nishimura, S., Inoue, T. and Sowa, M. Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. *Clin. Exp. Metastasis*, **14**, 43–54 (1996).
3) Yashiro, M., Chung, Y. S., Nishimura, S., Inoue, T. and Sowa, M. Establishment of two new scirrhous gastric cancer cell lines: analysis of factors associated with disseminated metastasis. *Br. J. Cancer*, **72**, 1200–1210 (1995).
4) Tahara, E. Growth factors and oncogenes in human gastrointestinal carcinomas. *J. Cancer Res. Clin. Oncol.*, **116**, 121–131 (1990).
5) Shimura, H., Date, K., Matsumoto, K., Nakamura, T. and Tanaka, M. Induction of invasive growth in a gallbladder cancer cell line by hepatocyte growth factor *in vitro*. *Jpn. J. Cancer Res.*, **86**, 662–669 (1995).
6) Gohji, K., Nakajima, M., Fabra, A., Bucana, C. D., von Eschenbach, A. C., Tsuruo, T. and Fidler, I. J. Regulation of gelatinase production in metastatic renal cell carcinoma by organ-specific fibloblasts. *Jpn. J. Cancer Res.*, **85**, 152–160 (1994).
7) van den Hooff, A. Stromal involvement in malignant growth. *Adv. Cancer Res.*, **50**, 159–196 (1988).
8) Morikawa, K., Walker, S. M., Nakajima, M., Pathak, S., Jessup, J. M. and Fidler, I. J. Influence of the organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res.*, **48**, 6863–6871 (1988).
9) Nakajima, M. and Chop, A. M. Tumor invasion and extracellular matrix degradative enzymes: regulation of activity by organ factors. *Sem. Cancer Biol.*, **2**, 115–127 (1991).
10) Fabra, A., Nakajima, M., Bucana, C. D. and Fidler, I. J. Modulation of the invasive phenotype of human colon carcinoma cells by organ specific fibroblasts of nude mice. *Differentiation*, **52**, 101–110 (1992).
11) Matsumoto, K., Date, K., Shimura, H. and Nakamura, T. Acquisition of invasive phenotype in gallbladder cancer cells via mutual interaction of stromal fibroblasts and cancer cells as mediated by hepatocyte growth factor. *Jpn. J. Cancer Res.*, **87**, 702–710 (1996).
12) Yashiro, M., Chung, Y.-S. and Sowa, M. Role of orthotopic fibroblasts in the development of scirrhous gastric carcinoma. *Jpn. J. Cancer Res.*, **85**, 883–886 (1994).
13) Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M. and McEvan, R. N. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.*, **47**, 3239–3245 (1987).
14) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–687 (1970).
15) Welch, D. R., Fabra, A. and Nakajima, M. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc. Natl. Acad. Sci. USA*, **87**, 7678–7682 (1990).
16) Ueki, N., Ohkawa, T., Yokoyama, Y., Maeda, J., Kawai, Y., Ikeda, T., Amuro, Y., Hada, T. and Higashio, K. Potentiation of metastatic capacity by transforming growth factor-β1 gene transfection. *Jpn. J. Cancer Res.*, **84**, 589–593 (1993).
17) Mooradian, D. L., McCarthy, J. B., Komanduri, K. V. and Furcht, L. T. Effects of transforming growth factor-β1 on human pulmonary adenocarcinoma cell adhesion, motility, and invasion *in vitro*. *J. Natl. Cancer Inst.*, **84**, 523–527 (1992).
18) Merzak, A., McCrea, S., Koocheckpour, S. and Pilkington, G. J. Control of human glioma cell growth, migration and invasion *in vitro* by transforming growth factor β1. *Br. J. Cancer*, **70**, 199–203 (1994).
19) Mahara, K., Kato, J., Terui, T., Takimoto, R., Horimoto, M., Murakami, T., Mogi, Y., Watanabe, N., Kohgo, Y. and Niitsu, Y. Transforming growth factor β1 secreted from scirrhous gastric cancer cells is associated with excess collagen deposition in the tissue. *Br. J. Cancer*, **69**, 777–783 (1994).
20) Guirguits, R., Marguies, I., Taraboletti, G., Schiffmann, E. and Liotta, L. Cytokine-induced pseudopodal protrusion is coupled to tumor cell migration. *Nature*, **329**, 261–263 (1987).
21) Liotta, L. A., Rao, C. N. and Barsky, S. H. Tumor invasion and the extracellular matrix. *Lab. Invest.*, **49**, 636–649 (1983).
22) Saksela, O., Moscatelli, D. and Rifkin, D. B. The opposing effects of basic fibroblast growth factor and transforming growth factor beta on the regulation of plasminogen activator activity in capillary endothelial cells. *J. Cell Biol.*, **105**, 957–963 (1987).
23) Weidner, K. M., Arakaki, N., Vandekereckhove, J., Weingart, S., Hartmann, G., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y. and Birchmeier, W. Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc. Natl. Acad. Sci. USA*, **88**, 7001–7005 (1991).
24) Rosen, E. M., Knesel, J., Goldberg, I. D., Jin, L., Bhargava, M., Joseph, A., Zitnik, R., Wines, J., Kelley, M. and Rockwell, S. Scatter factor modulates the metastatic

phenotype of the EMT6 mouse mammary tumor. *Int. J. Cancer,* **57,** 706–714 (1994).
25) Rong, S., Segal, S., Anver, M., Resau, J. H. and Vande Woude, G. F. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. *Proc. Natl. Acad. Sci. USA,* **91,** 4731–4735 (1994).
26) Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugiura, A., Tashiro, K. and Shimizu, S. Molecular cloning and expression of human hepatocyte growth factor. *Nature,* **342,** 440–443 (1989).
27) Takahashi, M., Ota, S., Shimada, T., Hamada, E., Kawabe, T., Okudaira, T., Matsumura, M., Kaneko, N., Terano, A., Nakamura, T. and Omata, M. Hepatocyte growth factor is the most potent endogenous stimulant of rabbit gastric epithelial cell proliferation and migration in primary culture. *J. Clin. Invest.,* **95,** 1994–2003 (1995).
28) Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M.-L., Kmiecik, T. E., van de Woude, G. F. and Aaronson, S. A. Identification of the hepatocyte growth factor receptor as the c-*met* proto-oncogene product. *Science,* **251,** 802–804 (1991).
29) Nardini, L., Vigna, E., Narsimhan, R., Guadino, G., Zarnegar, R., Michalopoulos, G. and Comoglio, P. M. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. *Oncogene,* **6,** 501–504 (1991).
30) Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H. and Tahara, E. Frequent amplification of the c-met gene in scirrhous type gastric cancer. *Biochem. Biophys. Res. Commun.,* **189,** 227–232 (1992).
31) Tahara, E., Kuniyasu, H., Yasui, W. and Yokozaki, H. Abnormal expression of growth factors and their receptors in stomach cancer. *Gann Monogr. Cancer Res.,* **42,** 163–173 (1994).
32) Lyons, R. M., Keski-Oja, J. and Moses, H. L. Proteolytic activation of latent transforming growth factor β from fibroblast conditioned medium. *J. Cell Biol.,* **106,** 1659–1665 (1990).
33) Horimoto, M., Kato, J., Takimoto, R., Terui, T., Mogi, Y. and Niitsu, Y. Identification of a transforming growth factor-β1 activator derived from a human gastric cancer cell line. *Br. J. Cancer,* **72,** 676–682 (1995).
34) Miyazono, K. and Heldin, C.-H. Role for carbohydrate structures in TGF-β1 latency. *Nature,* **338,** 158–160 (1989).
35) Odekon, L. E., Blasi, F. and Rifkin, D. B. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta. *J. Cell. Physiol.,* **158,** 398–407 (1994).