ANTICANCER RESEARCH *42*: 501-509 (2022)
doi:10.21873/anticanres.15508

Dipeptidyl Peptidase-4 from Cancer-associated Fibroblasts Stimulates the Proliferation of Scirrhous-type Gastric Cancer Cells

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Abstract. *Background/Aim: Cancer-associated fibroblasts (CAFs) may promote the malignancy of human scirrhous gastric cancer (SGC) cells. We conducted the present study to identify novel growth factors from CAFs. Materials and Methods: OCUM-12 and 2 CAF cell lines were used. The proliferation of cancer cells was determined by the number of cancer cells or the MTT assay. The growth factor(s) were purified and characterized by the gel filtration chromatography and protein array. Results: The molecular weight of the growth-stimulating factor was estimated to be approximately 66–669 kDa. Protein array of conditioned medium (CM) from CAFs indicated that dipeptidyl peptidase-4 (DPP-4) was one of the growth factors. The addition of CM increased the phosphorylation of C-X-C chemokine receptor 4 (CXCR4). The DPP-4 inhibitor significantly inhibited the growth-stimulating activity of CM. Conclusion: DPP-4 from CAFs might be one of the growth-stimulating factors for SGC through CXCR4.*

Human scirrhous gastric cancer (SGC) is characterized by rapid progression with abundant fibrosis. Macroscopic findings of SGC are diffuse infiltration, thick and hardened gastric wall. The microscopic finding of SGC is poorly differentiated adenocarcinoma. SGC accounts for ~10% of gastric cancers and has a poorer prognosis than other types of gastric cancers. It has been proposed that cancer-associated fibroblasts (CAFs) in the tumor microenvironment might play an important role in the progression of various types of cancer. We reported that CAFs might promote the malignant activity of SGC cells *via* an interaction between SGC cells and CAFs, which suggested that CAFs might produce growth-stimulatory factors for SGC cells (1). We clarified for the first time that fibroblast growth factor 7 (FGF7) produced from fibroblasts stimulated the proliferation of SGC cells *via* FGF7/fibroblast growth factor receptor 2 (FGFR2) signaling (2).

However, FGF7/FGFR2 signaling has been associated with only 20% of SGC cases (3, 4), which indicates that the other 80% of cases of SGC might be driven by signal(s) other than FGF7/FGFR2 signal in the interaction between SGC cells and CAFs. We conducted the present study to identify other growth factor(s) derived from CAFs for SGC cells without FGFR2 over-expression.

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Key Words: Cancer-associated fibroblasts, scirrhous-type gastric cancer, dipeptidyl peptidase-4, proliferation, cancer microenvironment.

Materials and Methods

Cell culture and cell lines. The culture medium was composed of Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan), 10% fetal bovine serum (FBS; Nichirei, Tokyo, Japan), 100 units/ml Penicillin-Streptomycin (Wako) and 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA). OCUM-12 (RRID: CVCL_8380), which is a human SGC cell line without FGFR2 over-expression established by us, was seeded in a 100-mm dish (Falcon, Lincoln Park, NJ, USA) and cultured in 10 ml of medium at 37˚C in a humidified atmosphere containing 5% CO_2 in air (5, 6). Two CAF cell lines, namely CAF100 and CAF64, were obtained from SGC and non-SGC, respectively. All human cell lines were authenticated using STR profiling. All experiments were performed with mycoplasma-free cells.

Preparation of conditioned medium (CM) from cancer-associated-fibroblasts (CAFs). Conditioned medium (CM) from fibroblasts was prepared, as follows. CAFs (5.0×10^4 cells/ml) were seeded into 100-mm plastic dishes with 10 ml of DMEM containing 5% FBS and

Table I. *Proteins for human XL cytokine array coordinates.*

	1, 2	3, 4	5, 6	7, 8	9, 10	11, 12	13, 14	15, 16	17, 18	19, 20	21, 22	23, 24
A	Reference spots	Adiponectin	Apolipo-protein A-I	Angiogenin	Angio-poietin-1	Angio-poietin-2	BAFF	BDNF	Complement component C5/C5a	CD14	CD30	Reference spots
B		CD40 ligand	Chitinase 3-like 1	Complement factor D	C-Reactive protein	Cripto-1	Cystatin C	Dkk-1	DPPIV	EGF	EMMPRIN	
C		ENA-78	Endoglin	Fas ligand	FGF basic	FGF-7	FGF-19	Flt-3 Ligand	G-CSF	GDF-15	GM-CSF	
D	GRO-α	Growth hormone	HGF	ICAM-1	IFN-γ	IGFBP-2	IGFBP-3	IL-1α	IL-1β	IL-1ra	IL-2	IL-3
E	IL-4	IL-5	IL-6	IL-8	IL-10	IL-11	IL-12 p70	IL-13	IL-15	IL-16	IL-17A	IL-18 Bpa
F	IL-19	IL-22	IL-23	IL-24	IL-27	IL-31	IL-32	IL-33	IL-34	IP-10	I-TAC	Kallikrein 3
G	Leptin	LIF	Lipocalin-2	MCP-1	MCP-3	M-CSF	MIF	MIG	MIP-1α/ MIP-1β	MIP-3α	MIP-3β	MMP-9
H	Myelo-peroxide	Osteopontin	PDGF-AA	PDGF-AB/BB	Pentraxin-3	PF4	RAGE	RANTES	RBP4	Relaxin-2	Resistin	SDF-1α
I	Serpin E1	SHBG	ST2	TARC	TFF3	TfR	TGF-α	Thrombo-spondin-1	TNF-α	uPAR	VEGF	
J	Reference spots		Vitamin D BP	CD31	TIM-3	VCAM-1						Negative controls

incubated for 3 days. The number of fibroblasts in each dish was approximately 2.5×10^6 cells after 3 days of incubation. To obtain CM, fibroblasts were washed twice with Dulbecco's phosphate-buffered saline (PBS) and then incubated for 3 days in 4 ml of DMEM. CM was collected from each dish and filtered through a 0.8 μm filter (Merck, Darmstadt, Germany). The supernatant was stored as CM at –20˚C until use. As a control, DMEM was used instead of CM. CM from CAF after co-culture of SGCs and CAFs was prepared as follows. CAFs and OCUM-12 were co-cultured in double chamber plates. OCUM-12 cells were seeded 5×10^4 on the upper layer of the membrane of pore size 0.45 μm (Kurabo, Osaka, Japan) and CAFs were seeded 1×10^4 on the lower chamber of a 24-well plate. Following incubation for 3 days, the upper layer was removed, and the culture supernatant was aspirated. Each well is washed with PBS, and then incubated with DMEM for 3 days to obtain CM. CM was collected from each well and filtered through a 0.8 μm filter. CM was stored at –20˚C until use.

Effect of CAF on the growth of gastric cancer cells. The proliferation of the gastric cancer cells was determined by calculating the number of cancer cells or by performing the MTT assay. The number of cancer cells was calculated following the addition of CM from CAF or the co-culture with CAF using a Coulter counter (Industrial D; Coulter Electronics, Luton, UK). MTT assay was performed as follow: 10 μl MTT solution obtained by diluting MTT (Dojindo, Kumamoto, Japan) with PBS to 5 mg/ml were added to each well and incubated for another 2 h. After incubation, the supernatant was removed and then 200 μl Dimethyl Sulfoxide (Wako) were added to each well. The plate was gently shaken until the formazan crystals were completely dissolved. Absorbance was measured at 570 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). To reveal the interaction between CAF and SGC, the proliferation assay was performed using a chamber with 0.45 μm porosity membrane filter (Kurabo) placed into a 24-well plate (Corning, Durham, NC, USA). Five-hundred μl of tumor suspension (2×10^4 cells per well) containing 1% FBS were added to the upper well and 500 μl of CAF suspension (2×104 cells per well) with 1% FBS were added to the lower well. Following incubation for 3 days, OCUM-12 cells in the upper chamber were collected and the number of cells was counted by a Coulter counter. As a control, only DMEM was added to the lower well and cancer cells were cultured in the upper well.

Gel filtration chromatography. The concentrated active fractions were applied to ACQUITY UPLC Protein BEH SEC Column (125 Å, 1.7 μm, 4.6 mm ×300 mm; Waters, Milford, MA, USA) equilibrated with PBS. The wavelength of the detector was set to 280 nm. The flow rate of the mobile phase was set to 0.3 ml/min. All procedures were carried out at room temperature. Thyroglobulin (MW 669,000), immunoglobulin G (MW 150,000), bovine serum albumin (MW 66,400), myoglobin (MW 17,000), ribonuclease A (MW 13,700) and uracil (MW 112) were used as the standard samples for the molecular weight calibration. The standard samples were purchased from Waters. An aliquot of 50 μl of OCUM-12 cell suspension (5×10^3 cells per well) with 2% FBS was inoculated into each well of 96-well plates (Corning) with a pulse of 50 μl of each fraction, and incubated. After 3 days, the MTT assay was performed to assess proliferation ability.

Proteome profiler protein array. Using the collected CM from single cultured CAFs or CAFs co-cultured with OCUM-12 cells, a total of 102 cytokines were measured using Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instruction (Table I). The chemiluminescence was detected by LAS-4000 mini (GE Healthcare, Pittsburgh, PA, USA) and quantified by ImageJ (7). Each quantified dot was divided by the average of the reference spots.

Effect of DPP-4 on the growth of OCUM-12 cells. We examined the effect of dipeptidyl peptidase-4 (DPP-4) (BioLegend, San Diego,

CA, USA) whose secretion from CAFs was enhanced after co-culture according to proteome profiler protein array. An aliquot of 100 μl of OCUM-12 cell suspension (5.0×10^3 cells/well) was inoculated into each well of a 96-well plate with various concentrations of DPP-4 (1, 10, 100 ng/ml), and incubated for 3 days. MTT assay was performed to assess proliferation.

Effect of DPP-4 inhibitor on the growth activity of DPP-4 and CM from CAF. Sitagliptin is a member of a class of drugs that inhibit DPP-4. We used Sitagliptin (Merck) to confirm a suppressive effect of DPP-4 on the growth activity. An aliquot of 100 μl of OCUM-12 cell suspension (5.0×10^3 cells/well) with 1% FBS and 100 ng/ml DPP-4 was inoculated into each well of a 96-well plate, and various concentrations of sitagliptin (10, 100, 1000 nM) were added. Following incubation for 3 days, the MTT assay was performed. As a control, OCUM-12 cell suspension was cultured in the absence of DPP-4 and sitagliptin. The effect of CM from CAFs on the proliferation of gastric cancer cells was determined by the MTT assay. Briefly, 50 μl of the tumor cell suspension (5×10^3 cells per well) with 2% FBS were added to 50 μl of CM in each well of 96-plates and incubated for 3 days. Then, the MTT assay was performed. As a control, 50 μl of DMEM were used instead of CM.

Effect of anti C-X-C chemokine receptor 4 (CXCR4) antibody on the effect of DPP-4 on the growth of cancer cells. We used an anti-CXCR4 antibody (R&D Systems) to confirm a suppressive effect of DPP-4 on the growth of cancer cells. An aliquot of 100 μl of OCUM-12 cell suspension (5.0×10^3 cells/well) with 1% FBS and DPP-4 100 ng/ml was inoculated into each well of a 96-well plate, and 2 μg/ml anti-CXCR4 antibody or 100 nM Sitagliptin were added. Following incubation for 3 days, the MTT assay was performed. As a control, OCUM-12 cell suspension was cultured in the absence of DPP-4, anti CXCR4 antibody, and sitagliptin. The proliferation activity of gastric cancer cells was determined by the MTT assay.

Western blot analysis. Cell lysates were collected after adding 50% CAF64 CM or CAF100 CM to the OCUM-12 culture medium and culturing for 3 days. After the protein concentration of each sample was adjusted, electrophoresis was carried out using 4 – 20% Mini PROTEAN TGX precast gels (Bio-Rad, Hercules, CA, USA). The protein bands obtained were transferred to Trans-Blot Turbo 0.2 μm poly vinylidene di-fluoride membrane (Bio-Rad). The membrane was kept in PBS-T (10 mM PBS and 0.05% Tween 20) supplemented with 0.1% membrane blocking agent at room temperature for 1 h. Then, the membrane was placed in PBS-T solution containing each primary antibody: CXCR4 (1:500, sc-53534 Santa Cruz Biotechnology, Dallas, TX, USA), Phospho-CXCR4 (pCXCR4, 1:500, CSB-PA060127, Cusabio Technology, Houston, TX, USA), and β-actin (1:5,000; Cell Signaling, Danvers, MA, USA) and allowed to react at room temperature for 2 h. The levels of specific proteins in each lysate were detected by autoradiography.

Statistical analysis. Collected data were statistically analyzed by Student's *t*-test. *p*-Value <0.05 was defined as statistically significant.

Results

The effect of the co-culture with CAFs on the proliferation of OCUM-12 cells. Figure 1A shows the growth-promoting activity of CAFs toward the scirrhous gastric cancer cell line OCUM-12, which does not show FGFR2 over-expression. The proliferation of OCUM-12 cells was significantly stimulated following co-culture with the cell lines CAF100 ($p=0.013$) or CAF64 ($p<0.0001$), compared to OCUM-12 cells alone. The co-culture with CAF100 significantly increased the number of OCUM-12 cells to 121% of the cell numbers in the control group, and the co-culture with CAF64 cells significantly increased the number of OCUM-12 cells to 138% of the cell numbers in the control group after 3 days culture.

The purification of growth-promoting factor(s) by gel filtration chromatography. The CM from CAF100 cells, which contains the growth-stimulating activity toward OCUM-12 cells without FGFR2 expression, was applied to an ACQUITY UPLC Protein BEH SEC Column. Fraction numbers 2, 3, and 4 showed significant proliferation-stimulating activity toward OCUM-12 cells compared to the control fraction. Among the three fractions, fraction number 2 showed the higher growth-stimulating activity of 132% compared to the other two fractions (Figure 1B). Considering the molecular weight of the polypeptide using the standard molecular markers, the molecular weight of the humoral factor present in fraction number 2 was estimated to be approximately 66-669 kDa.

Cytokines from CAFs. Figure 2A provides representative images of the protein array of CM from CAFs with or without the conditioning with OCUM-12 cells. Various cytokines and chemokines were observed to be produced from CAF100 and CAF64 cells (Figure 2B). Among them, DPP-4 was one of the cytokines identified in the conditioned medium of both CAF100 and CAF64 cells, and its relative ratio of their optical density was >0.2 when divided by the density of the reference spots after co-culture with SGC, and more than twice that of the single culture (Figure 3). We evaluated the effect of DPP-4 on the proliferation of scirrhous gastric cancer cell lines, as DPP-4 secretion from both CAF cell lines was increased by co-culture with scirrhous gastric cancer cell lines.

The effects of DPP-4 and the DPP-4 inhibitor sitagliptin on the proliferation of OCUM-12 cells. To determine whether DPP-4 is involved in the proliferation of OCUM-12 cells, we investigated the dose-response effect. OCUM-12 cells were cultured with 1 ng/ml, 10 ng/ml, or 100 ng/ml DPP-4. At 3 days after culturing, the growth of the OCUM-12 cells was significantly stimulated by the addition of DPP-4 at all three concentrations ($p<0.01$) (Figure 4A). Furthermore, 100 nM of the DPP-4 inhibitor sitagliptin significantly inhibited the effect of 100 ng/ml DPP-4 ($p<0.01$) (Figure 4B).

The effect of the DPP-4 inhibitor sitagliptin on the growth-stimulating effect of CM from CAFs on OCUM-12 cells. Both CAF CM promoted the proliferative activity of

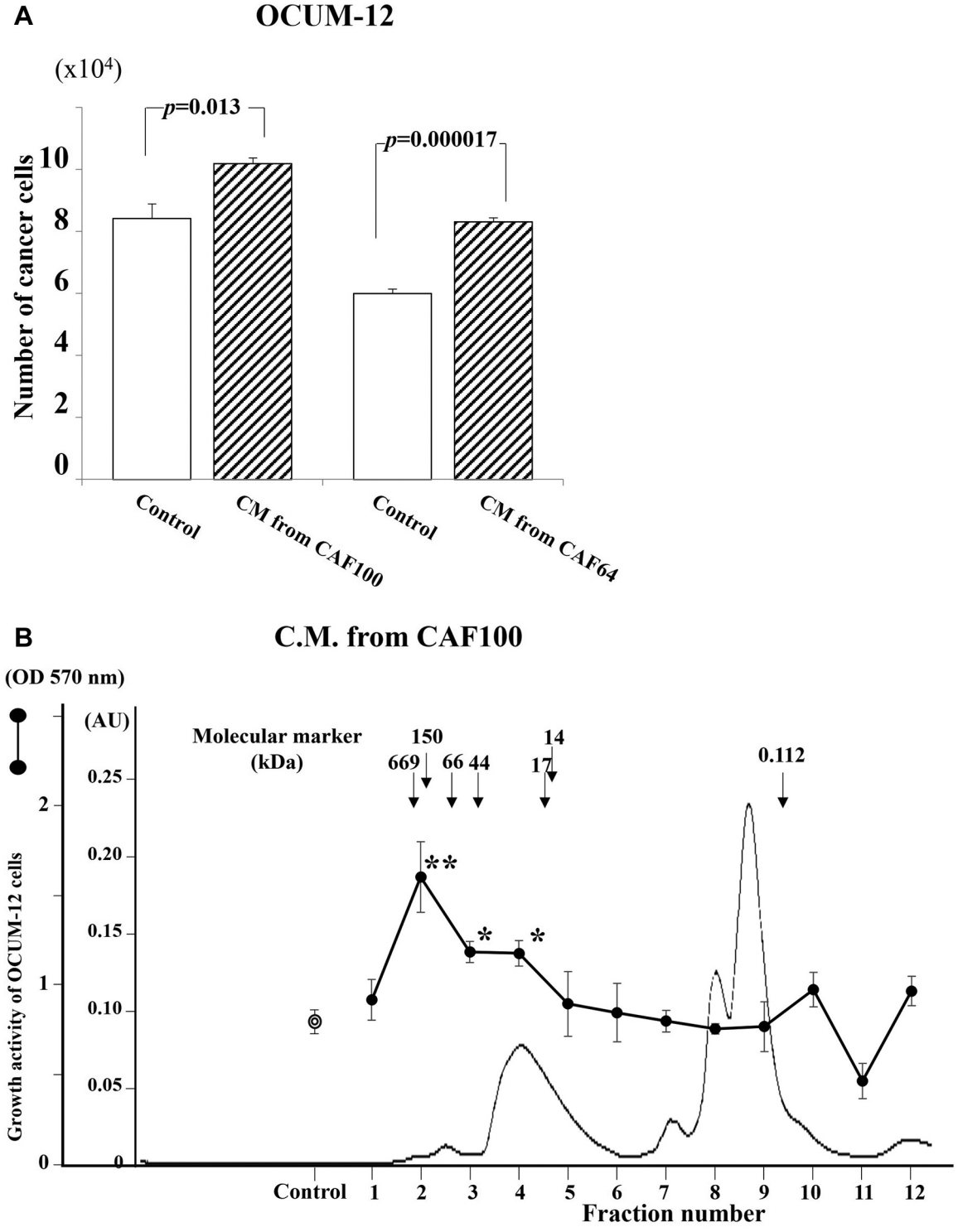

Figure 1. *Growth-promoting activity of cancer-associated fibroblasts (CAFs) for scirrhous gastric cancer (SGC) cells. (A) The effects of CAFs on the growth of scirrhous gastric cancer cells. The proliferation of OCUM-12 cells co-cultured with CAFs was significantly promoted compared to that of OCUM-12 cells alone. (B) Gel filtration chromatography results and the growth-promoting activity of the conditioned medium (CM) from CAF100 cells. The CM was applied to an ACQUITY UPLC Protein BEH SEC Column and eluted with PBS. The growth-promoting activity was determined by the MTT assay. The points are means of four samples. Bars: SD. *p<0.05, **p<0.01 compared to the control (open circle). The calculated molecular weight of the major peak was 150-669 p. Arrowheads indicate the positions of standard molecular markers: thyroglobulin (MW 669,000), immunoglobulin G (MW 150,000), bovine serum albumin (MW 66,400), myoglobin (MW 17,000), ribonuclease A (MW 13,700), and uracil (MW 112).*

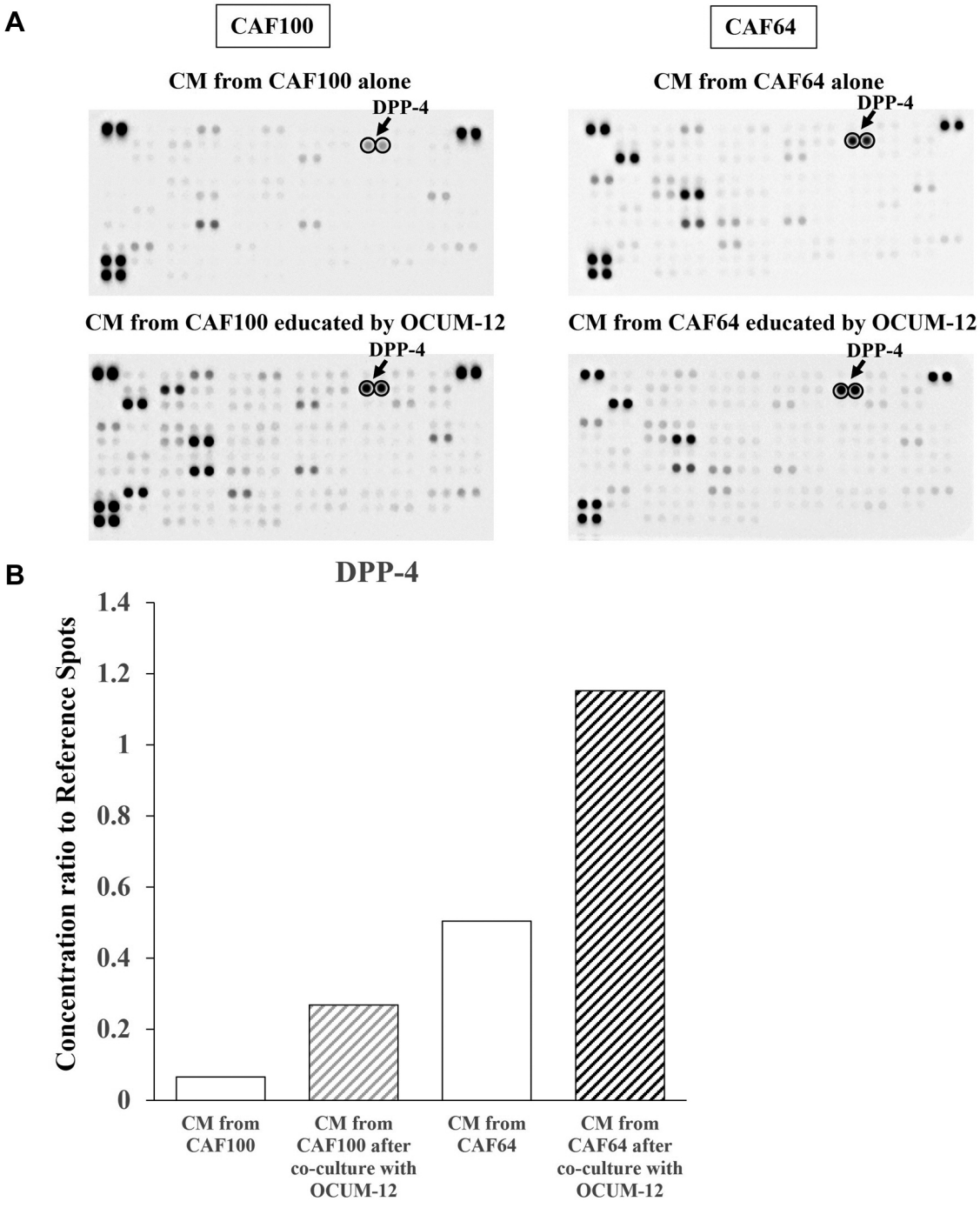

Figure 2. *Chemokines produced from cancer-associated fibroblasts (CAFs) with or without scirrhous gastric cancer (SGC) cells. (A) Profiles of proteins in the conditioned medium (CM) from CAFs. Antibodies of 105 cytokines were spotted on nitrocellulose membranes. Black circles: DPP-4 dots. (B) DPP-4; darker spots could be confirmed in CM from CAFs co-cultured with SGC cells compared to the single culture.*

OCUM-12, while 100 nM sitagliptin significantly inhibited the growth-stimulating effect of the CM from both cell lines, *i.e.*, CAF100 and CAF64, for OCUM-12 cells ($p<0.01$) (Figure 5A).

The effect of anti-CXCR4 neutralizing antibody on the growth-stimulating effect of DPP-4 on OCUM-12 cells. The anti-CXCR4 neutralizing antibody significantly decreased the growth-stimulating effect of DPP-4 on OCUM-12 cells (Figure 5B).

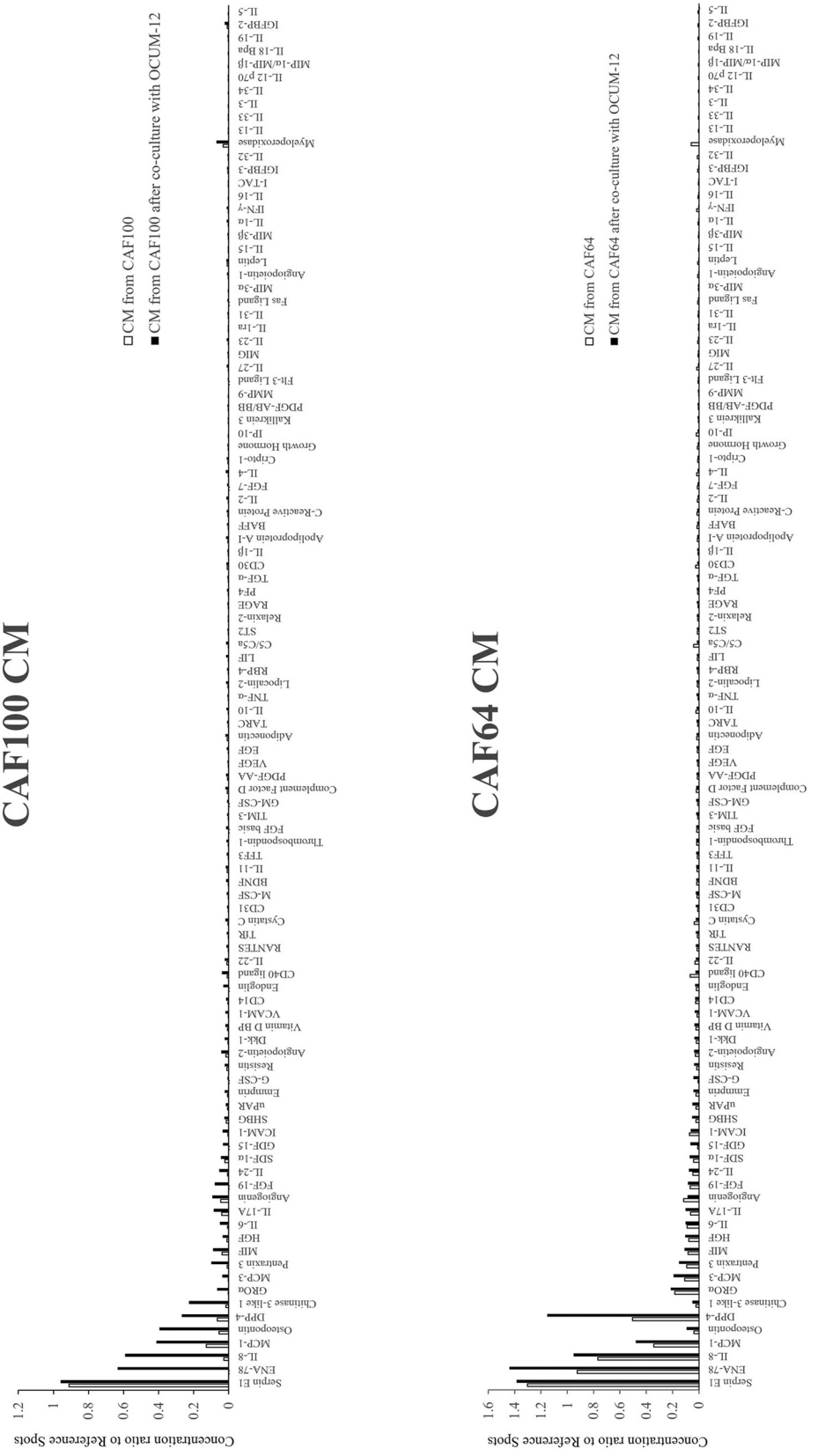

Figure 3. *Comparison of cytokines produced by CAFs. The dots of each cytokine obtained from the proteome profiler protein array were quantified and divided by the average of the reference spots. DPP-4 was one of the cytokines identified in the conditioned medium of both CAF100 and CAF64 cells, and its relative ratio of their optical density was >0.2 when divided by the density of the reference spots after co-culture with SGC, and more than twice that of the single culture.*

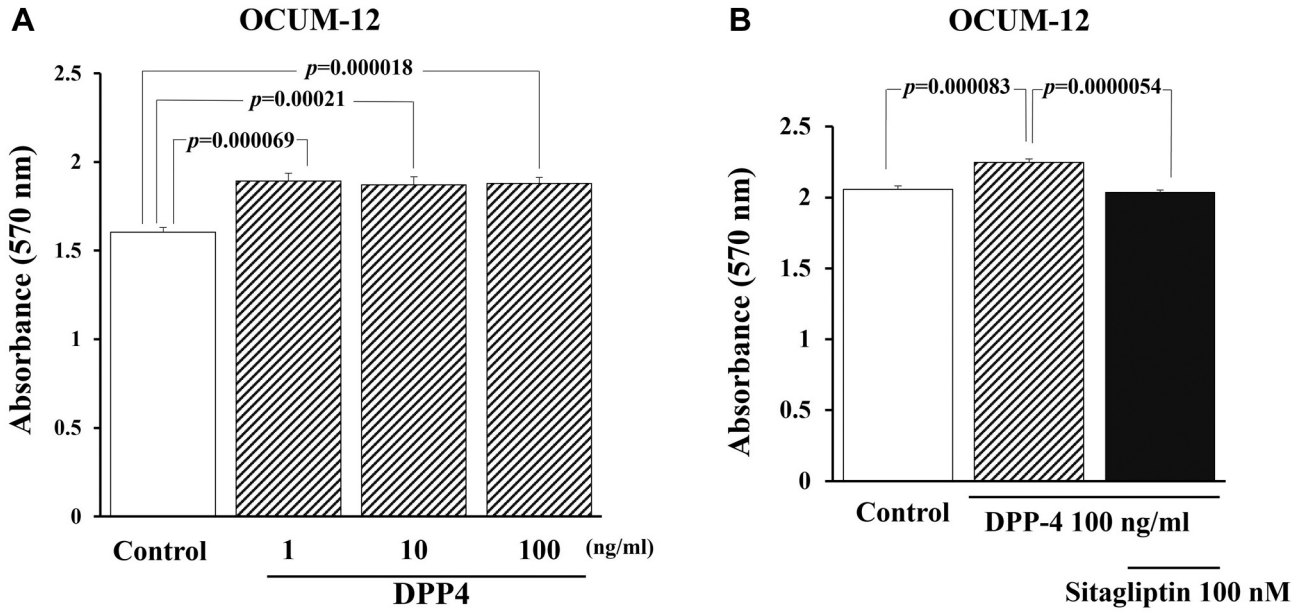

Figure 4. *The effects of DPP-4 and sitagliptin on the proliferation of scirrhous gastric cancer (SGC) cells. (A) DPP-4 promoted the growth of OCUM-12 cells (p<0.01). The results are shown as the average of 10 samples. Bars: standard error. The significance of differences was determined by Student's test. *p<0.01 compared to control. (B) The growth-promoting effect of DPP-4 on OCUM-12 cells was suppressed by sitagliptin (p<0.01). The results are shown as the average of 10 samples. Bars: SD.*

The effect of CAF CM on CXCR4 phosphorylation of OCUM-12 cells. The addition of CAF64 CM or CAF100 CM increased CXCR4 phosphorylation in OCUM-12 cells (Figure 5C).

Discussion

The results of our experiments demonstrated that DPP-4 from CAFs significantly promoted the growth of SGC cells. The DPP-4 inhibitor Sitagliptin suppressed the growth-stimulating activity of the CM from CAFs. The production of DPP-4 from CAFs was increased in the presence of SGC cells, resulting in the stimulation of the proliferation of SGC cells through the interaction between CAFs and SGC cells. It has been reported that DPP-4 and the number of DPP-4-positive fibroblasts were associated with each other and that DPP-4-positive fibroblasts expressed higher levels of myofibroblast markers (8). These findings suggested that DPP-4 might be one of the growth factors that is released from CAFs and affects proliferation of SGC cells without FGFR2 expression.

DPP-4 has also been reported to affect tumors. DPP4 expression has been confirmed in various tumors, and its expression levels correlate with tumor aggressiveness and invasiveness (9-11). Lam *et al*. demonstrated that DPP-4 expression is increased in colonic tumors, and they observed higher expressions of DPP-4 in tumors with higher TNM stages and with metastasis (12). These findings suggested that DPP4 from CAFs may play an important role in the malignant progression of SGC.

There are some recent reports on the tumor suppressor effects of DPP4 inhibitors on various carcinomas. Amritha *et al*. demonstrated that sitagliptin had an anti-cancer property against a colorectal cancer cell line (13). Jae-Hwi Jang *et al*. described an anti-tumor effect of the DPP-4 inhibitor vildagliptin that occurred *via* a down-regulation of autophagy that resulted in increased apoptosis and modulation of the cell cycle in colorectal lung metastases (14). These findings suggested that DPP-4 inhibitors might be promising therapeutic compounds for patients with SGC.

The addition of CM from CAFs increased the phosphorylation of CXCR4, a receptor of DPP-4. Taken together, the anti-CXCR4 neutralizing antibody significantly decreased the growth-stimulating activity of DPP-4 toward SGC cells. These findings suggested that DPP-4 from CAFs might stimulate the growth of SGC cells through CXCR4.

We previously reported that FGFR2 expression was suppressed and CXCR4 expression was enhanced in hypoxic conditions in diffuse-type gastric cancer cells (6). As a result, it suggested that diffuse-type gastric cancer cells might switch their driver pathways from FGFR2 signaling to SDF1-CXCR4 axis through HIF-1α in hypoxic tumor microenvironments. In this study, it was shown that DPP-4 may promote the

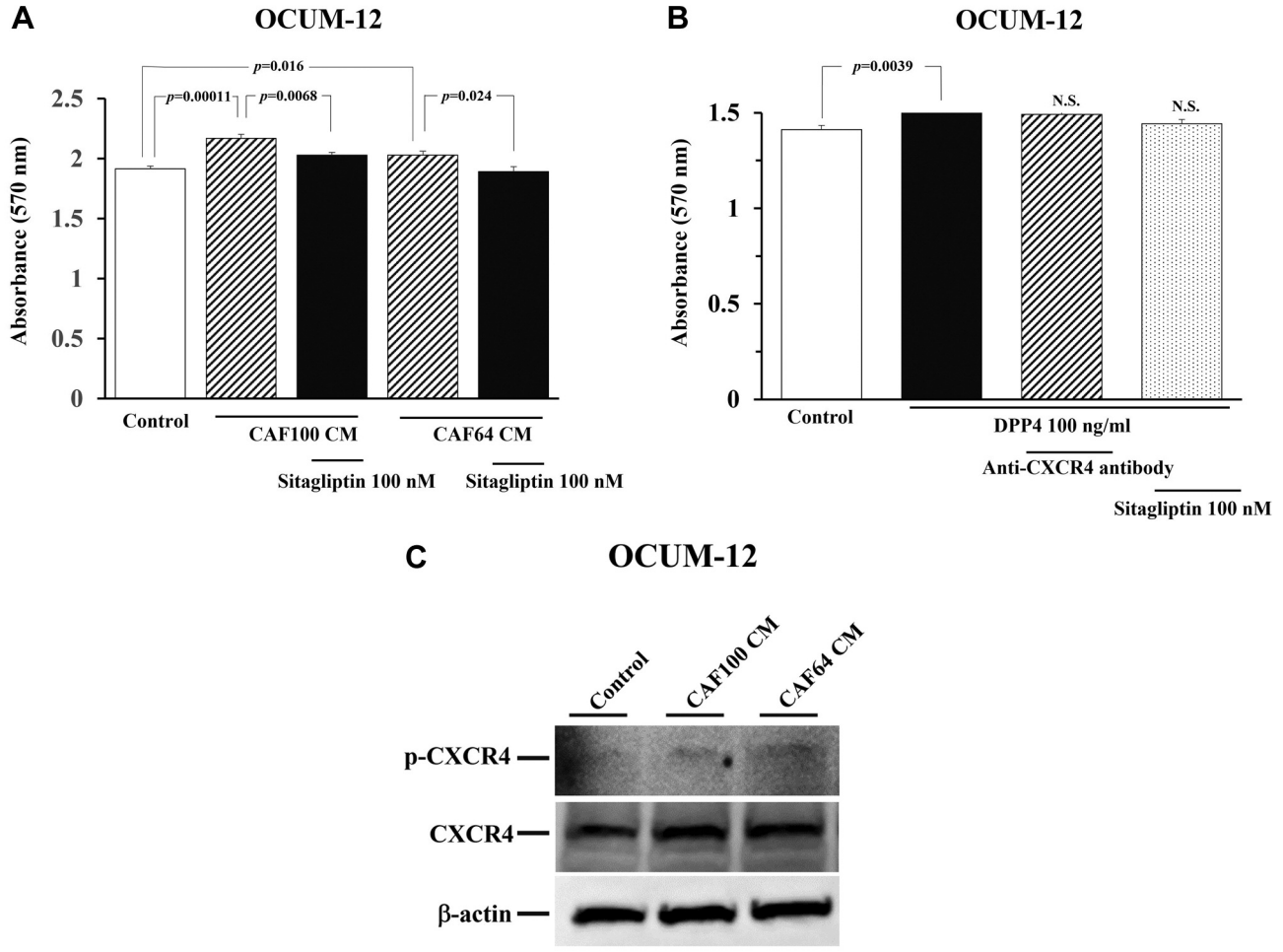

Figure 5. *The effects of DPP-4 signaling on the proliferation of scirrhous gastric cancer (SGC) cells. (A) The growth-inhibitory effect of sitagliptin on OCUM-12 cells incubated in the presence of the conditioned medium (CM) from cancer-associated fibroblasts (CAFs). OCUM-12 cells incubated with CM from CAFs exhibited significantly increased proliferative activity. When sitagliptin was added to OCUM-12 cells incubated with CM from CAFs, the growth-promoting effect of DPP-4 was suppressed. (B) The effect of the anti-CXCR4 antibody on the growth-stimulating effect of DPP-4 on OCUM-12 cells. When DPP-4 was added, the proliferative activity of OCUM-12 was promoted. When the CXCR4 antibody or Sitagliptin was added, DPP-4 did not promote the proliferative capacity of OCUM-12. (C) The effect of CAF CM on the expression of pCXCR4 and CXCR4 in OCUM-12 cells. Western blotting was performed in lysates of OCUM-12 cells cultured in the presence of CAF64 CM or CAF100 CM to examine the expression of CXCR4 and pCXCR4. The expression of CXCR4 was not affected. The expression of pCXCR4 was increased when both CAF64 CM and CAF100 CM were added compared with control.*

proliferative activity of SGC without FGFR2 over-expression by phosphorylating CXCR4. As a ligand for CXCR4, it was found that not only SDF1 but also DPP-4 is involved in the growth promoting signal. DPP-4 from CAFs might be one of the growth-stimulating factors for SGC *via* phosphorylation of CXCR4. The DPP-4 inhibitor Sitagliptin, might be a promising therapeutic compound for SGC.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

S.K. performed the experiments and wrote the manuscript; M.Y. designed and co-wrote the manuscript; Y.Y., T.S., and S.K.: sample preparation; A.S., S.N.: data accumulation; S.T., and K.K.: material sampling; M.O.: manuscript review. All Authors read and approved the final manuscript.

Acknowledgements

The Authors thank Akiko Tsuda (Molecular Oncology and Therapeutics, Osaka City University Graduate School of Medicine, Osaka, Japan) for technical support.

Funding

This study was partially founded by KAKENHI (Grant-in-Aid for Scientific Research; Nos. 18H02883 and 21H03008) from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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Received October 25, 2021
Revised November 20, 2021
Accepted November 22, 2021