Differential responses of scirrhouss and well-differentiated gastric cancer cells to orthotopic fibroblasts

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Summary Scirrhous gastric cancer cells proliferate rapidly with fibrosis, when the cancer cells invade into the submucosa of the stomach. To investigate the mechanisms responsible for the rapid proliferation, the growth interaction between gastric cancer cells and fibroblasts was examined. Human gastric cancer cell lines established from scirrhouss carcinoma or well-differentiated adenocarcinoma were used. Human fibroblast cell lines were obtained from various organs. The growth interaction between gastric cancer cells and fibroblasts was examined by calculating the number of cancer cells or by measuring [³H]thymidine incorporation of cancer cells. Gastric fibroblasts specifically stimulated the growth of scirrhouss gastric cancer cells, but not that of well-differentiated adenocarcinoma cells. The growth factor(s) produced from gastric fibroblasts were then partially purified and characterised. The growth-promoting factor(s) had apparent molecular weights of 10 000 dalton and was sensitive both to heat and proteinase treatment. No inhibition for the factor(s) was achieved with defined anti-growth factor antibodies. In this study, differential responses of scirrhous and well-differentiated gastric cancer cells to orthotopic fibroblasts were shown. Rapid proliferation of scirrhous gastric carcinoma should be partly controlled by orthotopic fibroblasts. The growth factor(s) from gastric fibroblasts, which was distinct from various defined growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), transforming growth factor-α (TGF-α), keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-I), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1) may play an important role in the progression of scirrhouss gastric cancer cells.

Keywords: scirrhouss gastric cancer; well-differentiated gastric cancer; fibroblast; growth interaction; growth factor

Human scirrhous gastric carcinoma (diffusely infiltrating carcinoma or Borrmann's type IV carcinoma) is characterised by cancer cell infiltration and proliferation with extensive fibrosis in the stroma (Tahara, 1990). Although the prognosis of gastric cancer has recently improved, that of scirrhous gastric cancer has not (Kiyasu et al., 1981). One of the reasons for the poor prognosis of this type of cancer is the difficulty of diagnosing it at an early stage, in part because of the rapid proliferation of the cancer cells. When scirrhous gastric cancer cells invade into submucosa of stomach, the cancer cells proliferate rapidly with fibrosis. The mechanisms responsible for the rapid proliferation are not understood clearly. The typical histological findings of scirrhous gastric carcinoma suggest that its development may be controlled by intercellular interactions between the cancer cells and the stroma cells such as fibroblasts. Recently, several studies have been published on the effect of fibroblasts on the production of the extracellular matrix of gastric cancer cells (Naito et al., 1984; Yamamoto et al., 1990). However, there has been no published report about the growth effect of gastric fibroblasts on scirrhous gastric cancer cells. Therefore, we examined the growth interaction between gastric cancer cells and fibroblasts derived from different organs, and partly purified and characterised a growth factor(s) for scirrhous gastric carcinoma.

Materials and methods

Cell types and cell culture

The human gastric cancer cell lines, OCUM-2M (poorly differentiated adenocarcinoma) (Yashiro et al., 1995), OCUM-1 (poorly differentiated adenocarcinoma), KATO-III (signet-ring cell carcinoma) (Sekiguchi et al., 1978), MKN-28 (well-differentiated adenocarcinoma) (Hojo, 1977), MKN-74 (well-differentiated adenocarcinoma) (Hojo, 1977), were cultivated in medium (see below) in a 100-mm culture dish (Falcon, Lincoln Park, NJ, USA), and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide in air. OCUM-2M, OCUM-1 and KATO-III were derived from scirrhous gastric carcinoma.

Human fibroblast cell lines were obtained from various organs. Original organ of each fibroblast cell line is presented in Table I. A fibroblast cell line, NF-8, and a scirrhous gastric cancer cell line, OCUM-2M, were obtained from the same patient (Yashiro et al., 1994). NF-1, NF-Eso, NF-Je and NF-Co were obtained from the same patient. The other fibroblast cell lines were derived from different patients. HS-27F was obtained from the American Type Culture Collection (Rockville, MD, USA), and the other fibroblast cell lines were derived from normal tissues of each organ in our laboratory, as follows. Briefly, each tissue specimen was excised under aseptic conditions and minced with forceps and scissors. Pieces of each tissue were cultivated in medium in a 100-mm culture dish (Falcon) and incubated in humidified incubators at 37°C in an atmosphere of 5% carbon dioxide and 95% air. The fibroblasts gradually grew in a monolayer. When confluent, the fibroblasts were collected and transferred to another culture dish every 5–7 days. The fibroblast origin was verified by immunostaining with two monoclonal antibodies against vimentin and human fibroblast (Dako, Glostrup, Denmark).

The culture medium was composed of Dulbecco’s modified Eagle medium (DMEM) (Bioproducts, Walkersville, MD, USA) with 2% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY, USA), 100 IU ml⁻¹ penicillin (ICN Biomedicals, Costa Mesa, CA, USA), 100 μg ml⁻¹ streptomycin (ICN Biochemicals), 2 mM glutamine (Bioproducts) and 0.5 mM sodium pyruvate (Bioproducts).
Table 1 Original organ of fibroblast cell line

| Fibroblast cell line | Original organ |
|----------------------|----------------|
| NF-8                 | Stomach        |
| NF-1                 | Stomach        |
| NF-Eso               | Oesophagus     |
| NF-Je                | Jejun          |
| NF-Co                | Colon          |
| NF-Liver             | Liver          |
| NF-Pa                | Parotis        |
| NF-Ma                | Mamma          |
| NF-Ov                | Ovary          |
| HS-27F               | Foreskin       |

Preparation of serum-free conditioned media

Serum-free conditioned medium (SF-CM) from fibroblasts was prepared as follows. 5.0 × 10^6 fibroblasts were seeded into 100-mm plastic dishes with 10 ml of DMEM containing 2% FCS, and incubated at 37°C for 3 days. To obtain the SF-CM, the fibroblasts were washed twice with Dulbecco’s phosphate-buffered saline (PBS) and then incubated for 2 days with 1 ml of DMEM. The number of fibroblasts in each dish was approximately 2 × 10^6 cells at the collection of SF-CM. The SF-CM was collected and centrifuged at 1000 g for 5 min, passed through filters (pore size 0.45 μm; Kurabo, Osaka, Japan) and stored at −20°C until use. The fibroblasts were used before the 15th passage in culture. Proliferative ability measured as doubling time was not different among the fibroblast cell lines.

Effect of fibroblasts 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 measuring [3H]thymidine incorporation.

The number of cancer cells was calculated following the addition of SF-CM from fibroblasts using a Coulter counter (Industrial D; Coulter Electronics, Luton, UK). To determine the optimal concentration of SF-CM for its growth-promoting activity, the kinetics and serum dependency of the activity produced from NF-8 cells were examined by culturing OCUM-2M cells. OCUM-2M cells were cultured in 24-well plates for 3 days in the presence of varying concentrations of SF-CM from NF-8 cells. Since the activity for OCUM-2M was evident following the addition of 25% SF-CM with 1–2% FCS (see Figure 1), the growth-promoting assay was conducted at 25% SF-CM. Briefly, 250 μl of SF-CM was added to 750 μl of tumour cell suspension (1 × 10^4 cells per well) with 2% FCS in each well of 24-well dishes, and incubated. The number of cells was counted at various time points using a Coulter counter. Serum-free medium instead of active fraction was used as a control.

The effect of fibroblasts on DNA synthesis of gastric cancer cells was determined by measuring [3H]thymidine incorporation. Briefly, 750 μl of the tumour cell suspension (1 × 10^5 cells per well) with 2% FCS was added to 250 μl of SF-CM in each well of 24-plates, and incubated with a pulse of 1 μCi per well of [3H]thymidine (28 Ci mmol⁻¹; Amersham, Tokyo, Japan) for 24 at 37°C. As a control, 250 μl of DMEM was used. The cells were then rinsed and collected on a membrane filter, and the radioactivity incorporated into DNA was determined in a liquid scintillation counter (Alokia, Tokyo, Japan).

Treatment of serum-free conditioned medium

The SF-CM obtained from NF-8 was used to characterise the growth-promoting activity. The growth activity was studied by culturing OCUM-2M cells as target. For measurement of heat stability of the growth-promoting activity, SF-CM was heated to 56°C for 30 min, 80°C for 10 min, and 100°C for 30 min. The susceptibility of the activity to proteases was examined by incubation of the SF-CM with 1 unit ml⁻¹ of proteases, trypsin (Sigma, St Louis, MO, USA), α-chymotrypsin (Sigma), or proteinase K (Sigma) at 37°C for 24 h. All samples were passed through filters (Kurabo). To determine whether the growth-promoting factor possessed heparin affinity, we examined the activity of SF-CM loaded onto the heparin affinity column ECHONO-Pac heparin cartridge (Bio-Rad, Richmond, CA, USA). Treated SF-CM (250 μl) was added to 750 μl of OCUM-2M cell suspension (1 × 10^5 cells per well) with 2% FCS in each well of 24-well dishes, and cultured for 3 days. The growth-promoting activity of the treated SF-CM was determined by calculating the number of OCUM-2M cells. The growth-promoting activity (% control) was calculated as:

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\text{Growth – promoting activity (% control)} = \frac{\text{number of cells cultured in medium with samples}}{\text{number of cells cultured in medium alone}} \times 100 - 100.
\]

Figure 1 Proliferative effects of conditioned medium from NF-8 cells on OCUM-2M cells. (a) OCUM-2M cells were cultured for 3 days in the presence of varying doses of the serum-free conditioned medium with 2% FCS. OCUM-2M cells were significantly increased following the addition of 10–50% SF-CM. (b) The growth of OCUM-2M cells with 25% SF-CM (●) was significantly increased in the presence of 0.3–20% FCS compared with control (■). The growth activity was evident with 1–2% FCS. The results are presented as the mean of three samples and the bars indicated the s.d. *P<0.05; **P<0.01 vs control.
**Ion-exchange chromatography**

The SF-CM from NF-8 was applied to a TSK-gel DEAE-5PW column (75 x 7.5 mm; Tosoh, Tokyo, Japan) equilibrated with PBS. The column was washed with 20 ml PBS and the bound protein was eluted with a linear gradient of 0–0.8 M sodium chloride in the PBS. Eluted protein was detected by UV absorption at 280 nm. All manipulations were carried out at room temperature. Aliquots of 250 μl of each fraction were added to 750 μl of OCUM-2M cell suspension (1 x 10^6 cells per well) with 2% FCS in each well of 24-well dishes, and growth-promoting activity was determined by calculating the number of cancer cells. Active fractions (20 ml) were then combined and concentrated to about 2 ml by ultrafiltration.

**Gel filtration chromatography**

The concentrated active fractions were applied to a TSK-gel G2000SXLW column (300 x 7.5 mm; Tosoh) equilibrated with PBS. The fractions were collected at a flow rate of 1 ml min^-1. The wavelength of the detector was set to 280 nm. All manipulations were carried out at room temperature. Ferritin (M, 450 000), bovine serum albumin (M, 67 000), ovalbumin (M, 45 000), cytochrome C (M, 12 300) and insulin chain B (M, 3500) were used as the standard samples for the molecular weight calibration. The standard samples were purchased from Serva Feinbiochemica, Heidelberg, Germany. An aliquot of 750 μl of OCUM-2M cell suspension (1 x 10^6 cells per well) with 2% FCS was inoculated into each well of 24-well plates (Falcon) with a pulse of 250 μl of each fraction, and incubated. After 3 days the number of cancer cells was counted.

**Effect of defined growth factors on the growth of OCUM-2M cells**

We examined the effect of various defined growth factors, including EGF (Gibco), b-FGF (Austral Biologics, San Ramon, CA, USA) KGF (UBI, Lake Placid, NY, USA), VEGF (Pepro Tec, Rocky Hill, NJ, USA), TGF-α (Becton Dickinson Labware, Mountain View, CA, USA), IGF-1 (Mallinckrodt, St Louis, MO, USA), PDGF-AA (Austral Biologicals), HGF (Becton Dickinson Labware), and TGF-β1 (King Brewing, Kakogawa, Japan) which are thought to affect the growth of gastric carcinoma (Yoshida et al., 1989, 1990; Yasui et al., 1988; Tanimoto et al., 1991; Hattori et al., 1994; Shibamoto et al., 1992; Ito et al., 1992) on the growth of OCUM-2M cells. An aliquot of 1 ml of OCUM-2M cell suspension (1.0 x 10^6 cells ml^-1) was inoculated into each well of 24-well plates (Falcon) with various concentrations of the defined growth factors, and incubated. The number of OCUM-2M cells was counted at various time points.

**Effect of anti-growth factor antibodies on the growth activity of conditioned medium from fibroblasts**

We used neutralising antibodies for several growth factors including anti-human EGF antibody (Oncogene Science, Uniondale, NY, USA), anti-human b-FGF antibody (Wako, Tokyo, Japan) (Hori et al., 1991), anti-TGF-α antibody (Pepro Tec), anti-HGF antibody (Sigma), rabbit IgG standard (Zymed, San Francisco, CA, USA) and mouse IgG standard (Tago, Burlingame, CA, USA). These antibodies were reconstituted in 0.1 g BSA per 100 ml of serum-free medium. Effect of the neutralising antibodies against their respective ligands was examined in experi-
mental study (see Table III). Antibody solutions of 4, 20, 100 and 400 μg ml⁻¹ were prepared and mixed 1:1 (v/v) with the peak growth activity fraction 22 by high-performance liquid chromatography (HPLC) (see Figure 3b). A 500 μl portion of OCUM-2M cell suspension (1 × 10⁶ cells per well) with 2% FCS was inoculated into each well of 24-well plates (Falcon). Antibody solutions (500 μl) were added to each well and incubated. Final solutions contained 25% fraction sample and antibody concentrations of 1, 5, 25 or 100 μg ml⁻¹. After 3 days the number of OCUM-2M cells was counted. Serum-free medium instead of active fraction was used as a control.

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

Results
Growth-promoting activity of serum-free conditioned medium from fibroblasts for various gastric cancer cells
The activity for OCUM-2M cells was evident following the addition of 25% SF-CM with 1–2% FCS (Figure 1); the

Figure 3 Effect of SF-CM from fibroblasts on DNA synthesis of gastric cancer cells. (a) The [³H]thymidine incorporation of OCUM-1 and KATO-III cells was significantly enhanced 130–180% by SF-CM from orthotopic fibroblasts (NF-1, NF-8), while that of MKN-28 and MKN-74 cells was not enhanced by SF-CM from any fibroblasts. (b) The [³H]thymidine incorporation of OCUM-2M cells was significantly enhanced 130–150% by SF-CM from orthotopic fibroblasts (NF-1, NF-8) compared with control, while that of OCUM-2M cells was not enhanced by SF-CM from various ectopic fibroblasts. The results are presented as the mean of three independent experiments and the bars indicate the s.d. *P<0.05; **P<0.01 vs control.
growth-promoting assay was then conducted at this concentration. The three scirrhous gastric cancer cell lines grew floating but not anchorage dependent in the culture medium and were not adherent to dishes following the

Table II  Biochemical characterisations of growth-promoting activity in the serum-free conditioned medium from NF-8 cells

| Heat treatment    | Growth-promoting activity (% control) | Inhibition (%) |
|-------------------|---------------------------------------|----------------|
| Untreated         | 32                                    |                |
| 56°C for 30 min   | 11                                    | 66             |
| 80°C for 10 min   | 2                                     | 93.7           |
| 100°C for 30 min  | 0                                     | 100            |

| Enzyme treatment  | Growth-promoting activity (% control) | Inhibition (%) |
|-------------------|---------------------------------------|----------------|
| Untreated         | 52                                    |                |
| Trypsin (1 unit ml⁻¹) | 0                                     | 100            |
| α-Chymotrypsin (1 unit ml⁻¹) | 0                                     | 100            |
| Proteinase K (1 unit ml⁻¹) | 0                                     | 100            |

| Heparin affinity  | Growth-promoting activity (% control) | Inhibition (%) |
|-------------------|---------------------------------------|----------------|
| Before heparin chromatography | 48                                    |                |
| After heparin chromatography   | 45                                    | 6              |

*Serum-free conditioned medium from NF-8 was subjected to different treatments as described in Materials and methods. The addition of the SF-CM. The SF-CM from gastric fibroblasts (NF-1, NF-8) significantly increased the number of scirrhous gastric cancer cells (OCUM-2M, OCUM-1, KATO-III) after 60 h in culture but not that of well-differentiated adenocarcinoma cells (MKN-28, MKN-74). The SF-CM from foreskin fibroblasts (HS-27F) did not increase the number of any cancer cells (Figure 2). The SF-CM from orthotopic fibroblasts (NF-1, NF-8) specifically stimulated the DNA synthesis of scirrhous gastric cancer cells by 130–180% compared with control, but not well-differentiated adenocarcinoma cells. The SF-CM from various ectopic fibroblasts did not stimulate the DNA synthesis of any type of gastric cancer cells (Figure 3).

Figure 4  Purification of the growth-promoting activity. (a) Cation-exchange chromatography. The SF-CM from NF-8 was applied to a TSK-gel DEAE-5PW column. Elution of protein was monitored by absorption at 280 nm. The growth-promoting activity of each fraction was examined by calculating the number of OCUM-2M cells. Peak activity was eluted at 160 mm sodium chloride, fractions 9 and 10. (b) Gel filtration chromatography of the SF-CM from NF-8. The SF-CM was applied to a TSK-gel G2000SWxlc column and eluted with PBS. The growth-promoting activity was determined by calculating the number of OCUM-2M cells. Calculated molecular weight of the major peak was 10000 dalton. Arrowheads indicate positions of standard molecular markers. Molecular weight markers included: ferritin (M₀ 450000), bovine serum albumin (M₀ 67000), ovalbumin (M₀ 45000), chymotrypsinogen A (M₀ 25000), cytochrome C (M₀ 12300) and insulin chain B (M₀ 3500).

Figure 5  Effect of defined growth factors on the growth of OCUM-2M cells. (a) OCUM-2M cells were cultured with various defined growth factors in concentrations ranging from 0.1–100 ng ml⁻¹, and then cell proliferation was determined by calculating the number of cancer cells after 72 h in culture. EGF, VEGF, TGF-α, KGF and b-FGF significantly stimulated OCUM-2M cell growth in concentrations ranging from 10–100 ng ml⁻¹ compared with control cells. IGF-I, PDGF and HGF had no significant effect on the growth of OCUM-2M cells. TGF-β decreased the growth of OCUM-2M cells. (b) OCUM-2M cells were cultured with 10 ng ml⁻¹ growth factors, and cell proliferation was determined at various time points. The growth of OCUM-2M cells was stimulated by EGF, VEGF, TGF-α, b-FGF and KGF after 48 h culture. The growth effect was evident after 72 h culture. Points, means of three samples and the bars indicate the s.d.
Characterisation of the growth-promoting activity

The effects of various treatments on the growth-promoting activity of the conditioned medium are shown in Table II. Protein concentrations in SF-CM from fibroblasts were measured by a Bio-Rad protein assay kit (Bio-Rad, Richmond, VA, USA) using BSA as a standard. Protein concentration in each SF-CM was 40.6–49.3 μg ml⁻¹ per 2 x 10⁶ cells. The activity was partially lost when heated at 56°C for 30 min and completely lost when heated at 80°C for 10 min. Treatment with trypsin, α-chymotrypsin, or protease K also destroyed the activity completely. The growth-promoting activity of SF-CM was retained even after heparin chromatography (Table II).

Purification of the growth-promoting activity

The SF-CM from NF-8 was applied to a TSK-gel DEAE-5PW column. Peak activity was eluted at 160 mM sodium chloride (Figure 4a). The active fractions 9 and 10 were concentrated and applied to a TSK-gel G2000SWXL column. A peak of growth-promoting activity was observed in fraction 22. From calculation of molecular weight of the polypeptide using the standard samples, it was estimated that the apparent molecular weight of the major peak was 10 000 dalton (Figure 4b). The growth of MKN-28 cells was not stimulated following the addition of fraction 22 (data not shown).

Effect of defined growth factors on the growth of OCUM-2M cells

To identify possible mitogens involved in OCUM-2M cell growth, we investigated the dose–response relationship between OCUM-2M cells and defined growth factors, including EGF, VEGF, TGF-α, IGF-I, KGF, b-FGF, PDGF-AA, HGF and TGF-β. OCUM-2M cells were cultured with these growth factors in concentrations ranging from 0.1–100 ng ml⁻¹. EGF, TGF-α and VEGF was significant stimulating OCUM-2M cell growth in concentrations ranging from 10 to 100 ng ml⁻¹ after 72 h culture. IGF-I, PDGF and HGF had no significant effect on the growth of OCUM-2M cells. TGF-β decreased the growth of OCUM-2M cells (Figure 5a). The growth effect was evident after 72 h culture (Figure 5b).

Effect of anti-growth factor antibodies on the growth activity of conditioned medium from gastric fibroblasts

To determine the relation between the growth activity of SF-CM and the defined growth factors which stimulated OCUM-2M cell growth, we tested whether neutralising antibodies against EGF, b-FGF and PDGF-AA were able to neutralise the growth-stimulating activity of the HPLC fraction 22. Bioactivity of each antibody in OCUM-2M cells was demonstrated in Table III. The growth activity of the fraction was not inhibited by any neutralising antibody (Table IV).

Discussion

In scirrhous gastric carcinoma, which is characterised by extensive carcinoma cell infiltration and proliferation with fibrosis, it is plausible that fibroblasts could affect the progression of the cancer cells from the standpoint of its characteristic histological findings. However, the growth interaction between gastric cancer cells and gastric fibroblasts has not been reported. In the present study, we have reported the organ-specific growth interaction between gastric cancer cells and fibroblasts. Besides NF-1 and NF-8, another five stomach-derived fibroblast cell lines also significantly stimulated the growth of scirrhous gastric cancer cells but not well-differentiated adenocarcinoma cells (data not shown). It was considered that gastric fibroblasts might specifically stimulate the growth of scirrhous gastric cancer cells in a

| Table III | Bioactivity of neutralising antibodies against 10 ng ml⁻¹ of EGF, TGF-α, b-FGF and VEGF |
|------------|-------------------------------------------------------------------------------------------------|
| Antibody   | Growth-promoting activity (%) | Bioactivity (%) |
| Anti-EGF antibody Control | 29 | 26 |
| | 1 μg ml⁻¹ | 26 |
| | 5 μg ml⁻¹ | 26 |
| | 25 μg ml⁻¹ | 9 |
| Anti-TGF-α antibody Control | 25 | 25 |
| | 10 μg ml⁻¹ | 24 |
| | 25 μg ml⁻¹ | 3 |
| | 100 μg ml⁻¹ | 38 |
| Anti-b-FGF antibody Control | 18 | 4 |
| | 1 μg ml⁻¹ | 78 |
| | 5 μg ml⁻¹ | 100 |
| | 25 μg ml⁻¹ | 100 |
| Anti-VEGF antibody Control | 26 | 26 |
| | 10 μg ml⁻¹ | 24 |
| | 25 μg ml⁻¹ | 6 |

*Neutralising antibodies were mixed with OCUM-2M cells which were cultured with each growth factor. The growth activity and bioactivity were determined for OCUM-2M cells as described in Materials and methods.

| Table IV | Effect of neutralising antibodies against EGF, TGF-α, b-FGF and VEGF on the growth activity of fraction 22a |
|----------|-------------------------------------------------------------------------------------------------|
| Antibody | Growth-promoting activity (%) | Inhibition (%) |
| Untreated | 78 | 0 |
| Mouse IgG | 1 μg ml⁻¹ | 80 |
| | 10 μg ml⁻¹ | 72 |
| | 25 μg ml⁻¹ | 70 |
| Rabbit IgG | 10 μg ml⁻¹ | 75 |
| | 25 μg ml⁻¹ | 74 |
| | 100 μg ml⁻¹ | 69 |
| Anti-EGF antibody | 1 μg ml⁻¹ | 76 |
| | 5 μg ml⁻¹ | 73 |
| | 25 μg ml⁻¹ | 66 |
| Anti-TGF-α antibody | 10 μg ml⁻¹ | 77 |
| | 25 μg ml⁻¹ | 71 |
| | 100 μg ml⁻¹ | 71 |
| Anti-b-FGF antibody | 1 μg ml⁻¹ | 80 |
| | 5 μg ml⁻¹ | 70 |
| | 25 μg ml⁻¹ | 68 |
| Anti-VEGF antibody | 10 μg ml⁻¹ | 79 |
| | 25 μg ml⁻¹ | 80 |
| | 100 μg ml⁻¹ | 80 |

*aNeutralising antibodies were mixed with the fractions 22 by HPLC, which stimulated the growth of OCUM-2M cells. The growth activity was determined for OCUM-2M cells as described in Materials and methods.
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Abbreviations

DMEM, Dulbecco’s modified Eagle medium; PBS, Dulbecco’s phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; EGF, epidermal growth factor; b-FGF, basic fibroblast growth factor; KGF, keratinocyte growth factor; VEGF, vascular endothelial cell growth factor; TGF-α, transforming growth factor alpha; IGF-1, insulin growth factor-I; PDGF-AA, platelet-derived growth factor AA homodimer; HGF, hepatocyte growth factor; TGF-β1, transforming growth factor-β1; HPLC, high-performance liquid chromatography.

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