Fibroblast Growth Factor-2 Accelerates Invasion of Oral Squamous Cell Carcinoma

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Abstract: The aim of this study was to examine the effects of fibroblast growth factor-2 (FGF-2) on cancer cell invasion and on fibroblast proliferation in an in vitro model of invasion. Three kinds of human oral squamous cell carcinoma cell lines with different invasive activity were used: OSC-20, OSC-19 (lower invasive type), and HOC313 (higher invasive type). FGF-2 and its high-affinity receptors FGFR-1 and FGFR-2 were detected by western blotting. The expression of FGF-2 and FGFRs mRNA was examined in cultured human oral squamous cell carcinoma cells by reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, recombinant human FGF-2 (rhFGF-2) was reacted with each cell line, and the invasion rate was determined by invasion assay. We also observed the behavior of cancer cell invasion in the collagen gel invasion model in the presence or absence of FGF-2-neutralizing antibody (anti-FGF-2). HOC313 cells showed higher expression of FGF-2 than OSC-20 and OSC-19 cells. The addition of rhFGF-2 promoted not only the proliferation of fibroblasts, but also the invasion of all cancer cell lines. In contrast, the addition of anti-FGF-2 completely inhibited the invasion of OSC-20 and OSC-19 cells. These results suggest that a higher invasiveness of squamous carcinoma cells is associated with higher production of FGF-2, which acts in an autocrine fashion to promote cancer cell invasion, and in a paracrine fashion to promote fibroblast proliferation.

Key words: fibroblast growth factor-2 (FGF-2), oral squamous cell carcinoma, invasion

Introduction

Many recent studies have investigated substances that are involved in the interaction between solid cancer cells and their induced fibroblasts1-6. Among them, basic fibroblast growth factor (FGF-2) was discovered by Gospodarowicz7 in 1974 as a protein that strongly promotes the proliferation of fibroblasts. Later studies reported its overexpression in highly malignant cancer cells, and malignant transformation of normal cells transfected with the FGF-2 gene8,9. These results suggest that FGF-2 is involved in the invasion of cancer cells and the proliferation of fibroblasts around cancer cells in an autocrine or paracrine fashion; however, the details are largely unknown.

Previously, we reported that in an immunohistochemical study of oral squamous cell carcinoma tissue, the expression or non-expression of FGF-2 in fibroblasts was associated with cancer invasion and metastasis, and the prognosis10.

In this study, we examined the effects of FGF-2 on cancer cell invasion and on fibroblast proliferation in an in vitro model of invasion.
Material and methods

1. Cell lines and culture methods

Three human oral squamous cell carcinoma cell lines with different invasive activities were used: OSC-20, OSC-19 (lower invasive type), and HOC313 (higher invasive type). OSC-20 is a cell line derived from a 58-year-old female with metastatic tongue cancer to the cervical lymph nodes. The mode of invasion was grade 3. OSC-19 is derived from a 61-year-old male with metastatic tongue cancer to the cervical lymph nodes. The mode of invasion was grade 4C. HOC313 is derived from a 51-year-old female with metastatic squamous cell carcinoma involving the mandibular gingiva and oral floor to the cervical lymph nodes. The mode of invasion was grade 4D (higher invasive type). In addition, the normal fibroblast line HGF-1 (ATCC, VA, USA) derived from the gingiva of a 28-year-old male, was used. Each cell line was cultured in Eagle’s minimum essential medium (Eagle-MEM) supplemented with 10% fetal bovine serum (FBS) (United Biotechnological, Victoria, Australia) and 2.5 mg/L of Fungizone (Bristol-Myers, NY, USA) at 37°C in an atmosphere of 5% CO2.

2. Western blot analysis

A 500 μL aliquot of M-PER (Pierce Biotechnology, Rockford, USA) was added to a culture of each cancer cell line and of fibroblasts, and the culture supernatant was separated by centrifugation. The concentration of protein in each supernatant sample was measured with a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA), and FGF-2 and its high-affinity receptors FGFR-1 and FGFR-2 were detected by western blotting. After SDS-PAGE, proteins were transferred onto a PVDF membrane, which was blocked for 12 h with 5% skim milk phosphate-buffered saline (PBS) containing 0.05% Tween 20. The membrane was reacted for 1 h with a 1: 200 PBS dilution of primary anti-FGF-2 polyclonal antibody SC-79 (Santa Cruz Biotechnology, CA, USA) or anti-FGFR-1 polyclonal antibody SC-121 (Santa Cruz Biotechnology, CA, USA) or anti-FGFR-2 polyclonal antibody SC-20735 (Santa Cruz Biotechnology, CA, USA), and subsequently with a 1: 1,000 PBS dilution of secondary anti-rabbit IgG antibody NA934 (Amersham, Tokyo, Japan) for 1 h. Protein expression was visualized with ECL-Western blotting detection reagents (Amersham International plc, Buckinghamshire, UK). To analyze the expression of FGF-2 in each cancer cell culture supernatant, cells from each cell line, grown to confluence in a 100-mm tissue culture dish, were washed with PBS and cultured in serum-free Eagle-MEM containing 0.1% BSA for a further 24 h. The culture supernatant was separated by centrifugation, and FGF-2 was detected by western blotting.

3. Analysis of expression of FGF-2 and FGFRs mRNA

The expression of FGF-2 and FGFRs mRNA was examined in cultured human oral squamous cell carcinoma cells by reverse transcriptase polymerase chain reaction (RT-PCR). RNA was extracted from OSC-20, OSC-19, and HOC313 cells using an RNeasy Kit (QIAGEN KK, Tokyo, Japan). Single-strand cDNA was synthesized from the RNA, and DNA was amplified by PCR with FGF-2- or FGFRs-specific primers. The PCR products were electrophoresed on agarose gels, and analyzed by photography under UV illumination. The primers and PCR conditions used are shown in Table 1.

4. Fibroblast proliferation

Fibroblast suspensions (3 × 10⁵ cells/ml) were pre-
pared with serum-free Eagle-MEM containing 0.1% BSA and 0, 10, 50, 100, 500, or 1,000 ng/ml of recombinant human FGF-2 (rhFGF-2) (PEPRO TECH EC LTD., London, UK). Aliquots of 100 μL (3 × 10⁴ cells) of these cell suspensions were placed in wells of 96-microwell plates, incubated at 37°C in a 5% CO₂ atmosphere for 3 d, and reacted with the cell detection reagent (CellTiter96AQueous One Solution Reagent). The proliferative activity of the cells was determined by their absorbance at 490 nm.

5. Invasion assay

BD BioCoat Matrigel Invasion Chambers (8-μm pore-size PET membrane, 24 wells; BD Biosciences, MA, USA) were used to assess the invasive ability of each cancer cell line. Suspensions (5 × 10⁴ cells/ml) of each cancer cell line were prepared with serum-free Eagle-MEM containing 0.1% BSA. A 500 μL suspension (2.5 × 10⁴ cells) of each cancer cell line was placed in the upper chamber of an Invasion Chamber, 750 μL of rhFGF-2 (50 ng/ml) was added to the lower chamber, and the whole chamber was incubated at 37°C in a 5% CO₂ atmosphere for 24 h. Under a light-microscope (× 100 magnification), cells observed on the undersurface of the membrane were counted as invading cells. A similar procedure was performed using a BD BioCoat Control Insert (8-μm pore-size, no ECM, 24 wells; BD Biosciences), and cells observed on the undersurface of the membrane were counted as migrating cells. The invasion rate¹⁶,¹⁷ of each cell line was calculated, and compared with that of each cell line cultured in the absence of rhFGF-2.

6. Collagen gel invasion model

Collagen gels were used as matrices for cancer cell invasion, and prepared according to the method of Wren et al.¹⁸. Type I collagen (Cellmatrix type I-A; Nitta Gelatin Inc., Tokyo, Japan), 10-fold concentrated EMEM, and reconstituting buffer (0.05 N NaOH solution containing 200 mM HEPES and 2.2% NaHCO₃) were mixed at a ratio of 8:1:1, and allowed to gel while enclosing 3 × 10⁴ fibroblasts per ml. Cancer cells (2 × 10⁶) from each cell line were overlaid on a collagen gel, and 3 ml of Recombinant Human FGF-2 (rhFGF-2) (50 ng/ml) in serum-free Eagle-MEM containing 0.1% BSA was added. The cancer cells were cultured in suspension at 37°C in a 5% CO₂ atmosphere for 21 d. When the cell culture was completed, the rate of collagen gel contraction relative to the contraction of the control gel containing no rhFGF-2 was determined. The collagen gel was fixed in 10% formalin, embedded in paraffin, stained with hematoxylin and eosin by standard techniques, and observed for cancer cell invasion.

7. Immunohistochemical examination

Cancer cells (2 × 10⁶) from each cell line were overlaid on a collagen gel, and 3 ml of FGF-2-neutralizing antibody (anti-FGF-2) (R & D Systems, MN, USA) (5 μg/ml) in 10% FBS-supplemented Eagle-MEM containing rhFGF-2 (50 ng/ml) was added. The cancer cells were cultured in suspension at 37°C in a 5% CO₂ atmosphere for 14 d. Each specimen was fixed in 10% buffered formalin, and then embedded in paraffin to prepare serial sections (4 μm). After treating with trypsin solution for 30 min at 37°C, immunohistochemical staining was performed by the Labeled Strept Avi-din-Biotin method following deparaffinization and rehy-dration. As a primary antibody, rabbit polyclonal anti-bFGF antibody (SC-79) (× 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was reacted at 4°C for 24 h. The slides were incubated with biotinylated anti-rabbit Ig (DACO, Kyoto, Japan), and reacted at room temperature for 60 min. After reacting with peroxidase-conjugated streptavidin (DACO) for 60 min, they were washed with PBS. Immunohistochemical reactions were developed in 3,3’-diaminobenzidine tetrahydrochloride (Wako, Osaka, Japan), then counterstained with hematoxylin.

Results

1. Expression of FGF-2, FGFR-1 and FGFR-2

Western blot analysis of FGF-2, FGFR-1 and FGFR-2 proteins showed that the proteins were uniformly expressed in each cancer cell line and HGF-1. The analysis of FGF-2 protein in the culture supernatant of each cancer cell line revealed strong expression of FGF-2 protein in HOC313, but no expression of FGF-2 protein in the culture supernatant of HGF-1 (Fig. 1).

2. Expression of FGF-2 and FGFRs mRNA in cultured human oral squamous cell carcinoma cells

FGF-2 mRNA was expressed in all cancer cells. Among the FGFRs mRNA, FGFR-1 mRNA was ex-
pressed weakly in OSC-20 and strongly in HOC313, whereas FGFR-2 mRNA was expressed to a similar degree in all cancer cells (Fig. 2).

3. Effects of FGF-2 on fibroblast proliferation

We examined the optimal concentration of rhFGF-2 for fibroblast proliferation by absorbance spectrometry, and found that the absorbance increased abruptly at the concentration of 50 ng/ml. At rhFGF-2 concentrations of 100, 500, and 1,000 ng/ml, the absorbance was almost constant at 0.875 (Fig. 3).

4. Effects of FGF-2 on cancer cell invasion

We examined the effects of rhFGF-2 (50 ng/ml) on the invasion of each cancer cell line, and found that the invasion rate was higher in the presence than in the absence of rhFGF-2. In particular, the addition of rhFGF-2 (50 ng/ml) significantly increased the invasion rate of OSC-19 from 0.56% to 7.4% (P < 0.05, Fig. 4).

5. Contraction of collagen matrices

When each cancer cell line was cultured in rhFGF-2-containing medium, the collagen matrix markedly contracted. In particular, culture of OSC-20 cells resulted in contraction rates of 54.6% and 8.3% in the presence and absence (control) of rhFGF-2, respectively (P < 0.05, Fig. 5).
Fig. 3  Effects of FGF-2 on fibroblast proliferation
The absorbance of fibroblast cultures in the presence of 50 ng/ml of rhFGF-2 markedly increased over that of fibroblast cultures in the absence of rhFGF-2.

Fig. 4  Invasion rate of each cell line in the presence of FGF-2
A, B: Light-microscopic findings of the base surface of the Boyden Chamber after invasion assay by Giemsa stain. Invasion rate = (Number of invading cells/Number of migrating cells) × 100. The addition of rhFGF-2 (50 ng/ml) significantly increased the invasion rate of OSC-19 and HOC313 (Mann-Whitney test).
6. Cancer cell invasion in collagen matrices

We observed the behavior of cancer cell invasion in the collagen gel invasion model in the presence or absence of the rhFGF-2. In the presence of rhFGF-2, OSC-20 cells invaded in aggregates into the matrix; OSC-19 cells extended projections in cords into the matrix, and were in contact with many spindle-shaped fibroblasts; HOC313 cells were in contact with fibroblasts, and invaded diffusely (Fig. 6).

7. Effects of anti-FGF-2 in collagen matrices

The addition of anti-FGF-2 completely inhibited the invasion of OSC-20 and OSC-19 cells. Cancer cells and fibroblasts were immunohistochemically examined for the expression of FGF-2 in a collagen gel invasion model containing anti-FGF-2 or no anti-FGF-2 (control). FGF-2 was expressed in cancer cells in both models, and in fibroblasts accumulated at the invasive front of cancer cells in the control model. However, no FGF-2 expression was observed in fibroblasts accumulated at the cancer invasive front in the anti-FGF-2-containing model (Fig. 7).

Discussion

The invasion of cancer cells is associated with tumor stromal fibrosis\(^\text{19,20}\), and cancer cells are considered to
interact with the fibroblasts proliferating around cancer cells at the invasive front through cancer cell-produced factors, thereby promoting cancer cell proliferation and invasion. It has been reported that cancer cells may produce several factors such as plasminogen activator and tumor-derived growth factor; however, factors
linking the proliferation and invasion of cancer cells to the proliferation of host fibroblasts have not been clearly identified, and their actions are largely unknown. Among the growth factors overexpressed in cancer cells, fibroblast growth-promoting FGF-2 was found to be strongly expressed in cancer cells and fibroblasts at the invasive front in our previous immunohistochemical study. Myoken et al. examined oral squamous cell carcinomas by immunohistochemical techniques, and reported stronger expression of FGF-2 than that in normal tissue, suggesting the possibility of its involvement in malignant transformation and self-proliferation of cells. Furthermore, additional studies have reported high expression of FGF-2 in human malignant tumor cell lines, and that fibroblasts transfected with FGF-2 cDNA underwent malignant transformation, acquiring self-proliferative ability. Western blot analysis confirmed the expression of FGF-2, FGFR-1, and FGFR-2 proteins in three cancer cell lines with different modes of invasion. FGF-2 was also detected in the culture supernatant of each cancer cell line, particularly in that of HOC313. Since the culture supernatant of HGF-1 did not show expression of FGF-2, this result suggests that normal fibroblasts do not secrete FGF-2 extracellularly, unless they receive some stimulus. Furthermore, examination by RT-PCR of each cancer cell line for the expression of mRNA for FGF-2 and FGFRs showed strong expression of FGFR-1 mRNA in HOC313 cells, suggesting that cancer cells with a higher invasive potential express higher levels of FGF-2 and its receptors. Schultz-Hector et al. and Kramer et al. reported that the higher proliferative activity of oral squamous cell carcinomas was associated with higher expression of FGF-2, which increased the production of cancer cell proteases, thereby enhancing cancer cell invasive activity, suggesting an association between FGF-2 production and oral malignant tumor cell invasiveness. Although FGF-2 has been reported to promote the invasion of OSC-20 and OSC-19 (lower invasive type) in the presence of fibroblasts, the invasion assay showed that the addition of rhFGF-2 promoted the invasiveness of cancer cells even in the absence of fibroblasts. In addition, the collagen gel invasion model of each cancer cell line showed a high rate of gel contraction in the presence of rhFGF-2, with prominent invasion of cancer cells, suggesting that cancer cells produce FGF-2, which acts on them in an autocrine manner, thereby being involved in their invasiveness. On the other hand, we examined each cancer cell line for changes in invasive behavior following the addition of anti-FGF-2, and found that it completely inhibited the invasion of OSC-20 and OSC-19 cells. In addition, immunohistochemical examination of FGF-2 expression showed that neutralization treatment with anti-FGF-2 caused loss of FGF-2 expression in fibroblasts accumulated around cancer cells. These findings suggest that the inhibition of fibroblast proliferative activity reduces the invasiveness of cancer cells. The results of this study indicate that FGF-2 produced by cancer cells promotes their own invasion in an autocrine fashion, and simultaneously promotes the proliferation of the surrounding fibroblasts in a paracrine fashion; thus, the interaction between cancer cells and fibroblasts is involved in the invasion of cancer cells. Finally, a squamous cell carcinoma cell line with higher invasive potential showed higher expression of FGF-2, suggesting that the level of FGF-2 expression is an indicator of the degree of cancer malignancy.

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