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Primary Human Fibroblasts Induce Diverse Tumor Invasiveness: Involvement of HGF as an Important Paracrine Factor

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Fibroblasts have been considered to play an important role in tumor progression. In order to evaluate the contribution of fibroblasts to tumor invasion, TE2-NS, an esophageal cancer cell line, was cultured on collagen gel containing primary fibroblasts derived from normal esophageal submucosa or cancerous tissues of seven esophageal cancer patients. TE2-NS showed diverse invasiveness into the underlying gel containing fibroblasts, but did not invade the gel not containing fibroblasts. The invasiveness of TE2-NS, which expressed hepatocyte growth factor (HGF) receptor, was well-correlated with the concentration of HGF in conditioned medium. Administration of neutralizing antibody against HGF effectively suppressed the invasion, but application of recombinant HGF without fibroblasts induced little invasion into the gel. Fibroblasts from non-cancerous tissue generally secreted a larger amount of HGF and induced tumor invasion to a greater extent than those from cancer tissue, with large diversity. Interestingly, HGF secretion of fibroblasts from non-cancerous tissue was stimulated by co-culture with TE2-NS in two lines, but not in the other four. These results indicate that HGF is an important paracrine factor which induces tumor cell invasion, and the diversity of HGF production by fibroblasts might suggest different potentiality to induce tumor invasion among patients.

Key words: Hepatocyte growth factor — Primary fibroblast — Esophageal cancer — Tumor invasion — Organotypic raft culture

It has been reported that fibroblasts or paracrine factors produced by fibroblasts, are deeply involved in the regulation of cancer cell behavior including proliferation, invasion and metastasis. Growth and metastatic capacity of various epithelial tumor cell types in human have been reported to be enhanced when the cells are injected into the animals admixed with fibroblasts.[1–3] Interactions between human cancer cells and fibroblasts have been investigated using primary cultured human fibroblasts obtained from different organs.[4,5] For example, Matsumoto *et al.* have also shown that the *in vitro* invasion of squamous cell carcinoma into a collagen gel can be substantially augmented in the presence of fibroblasts.[6] However, the mechanisms of the invasion induced by fibroblasts are not clearly understood.

To evaluate the characteristics of cancer cell invasion *in vitro*, we have been using organotypic raft culture in which non-invasive tumor cells can reconstitute stratified epithelium, but invasive tumor cells will invade the underlying collagen/fibroblast matrix. In this system, esophageal cancer cells with E-cadherin dysfunction showed invasive characteristics.[7,8] Interestingly, this E-cadherin-related invasiveness of the tumor was variable when different fibroblast cell lines were used in raft culture.[7] The most aggressive invasion was observed when MRC-5 fibroblasts were used. This observation implies that some soluble molecule(s) secreted by fibroblasts might affect the invasiveness evaluated using this system. In the present study, we examined the *in vitro* invasiveness of an esophageal cancer cell line induced by primary human fibroblasts derived from non-cancerous submucosal tissues or cancer tissues of esophageal cancer patients using the organotypic raft culture. Furthermore, we examined the correlation between hepatocyte growth factor (HGF) production by primary fibroblasts and tumor invasiveness in this model, since MRC-5 fibroblast cell line, which was the most potent inducer of cancer cell invasion in a previous study,[7] is known to express HGF at a high level.

MATERIALS AND METHODS

Cell culture TE2 is a cell line established from squamous cell cancer of human esophagus (kindly provided by Dr. T. Nishihira, Tohoku University Medical School). TE2-

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NS was cloned from the TE2 cell line using the limiting dilution method described previously.[9]

Primary fibroblasts were isolated from seven esophageal cancer patients, who underwent esophagectomy in the Department of Surgery II, Osaka University Medical School or the Department of Surgery, Kinki-chuo Hospital. No patient had received anticancer therapy prior to the operation. The histological diagnosis of the tissues was determined by standard histopathological investigations. The pathologic classification was based on TNM classification of malignant tumors.[10] The items assessed in this study were tumor depth, lymph node status, distant metastasis, and histologic grade of tumor.

Fresh tissue samples were obtained from malignant esophageal tumors and non-cancerous submucosal tissues distant from the tumor in the same patients. The tissues were minced into pieces of about 1 mm^3, which were incubated at 37°C for 2 h in growth medium (F/M-medium: a 1 : 1 mixture of HAM F12 and DMEM medium containing 2 m*M* glutamine, 10 m*M* $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin) supplemented with 1000 U/ml dispase (Godo Shusei, Tokyo). The digested tissue was centrifuged at 1000 rpm for 5 min, then the cells were resuspended and cultured to subconfluence in growth medium containing 10% fetal bovine serum at 37°C in 5% CO_2 in air during 1–2 weeks with periodic medium changes. Cells were trypsinized and transferred to new flasks when they were subconfluent. After 4 to 5 passages, the resultant cultured cells were examined by light microscopy to confirm that they were fibroblasts without epithelial cell contamination.

Invasion assay with organotypic raft culture *In vitro* tumor invasiveness was evaluated using the procedures described previously.[7] In 12-well plates, 1×10^5 tumor cells suspended in 1 ml of medium were seeded on gel containing 1×10^6 primary cultured fibroblasts and 1.0 mg/ml type I collagen, Cell Matrix Type I-A (Nitta Gelatin, Yao) in 3 ml of F/M medium. After 24 h incubation, the gels were detached from the well, incubated for an additional 24 h to induce contraction of the gel, and floated at the air-fluid interface on stainless steel grids placed in a 100-mm culture dish. Ten ml of F/M medium containing 10% fetal calf serum was fed every three days. Culture medium and gel were supplemented with either 10 ng/ml neutralizing antibody (Ab) against HGF[11] or the same concentration of non-immunized rabbit serum (Cedarlane Laboratories, Hornby, Canada) to inhibit invasion. Ten μg/ml or 50 μg/ml recombinant HGF (rHGF),[12, 13] and/or 30 μg/ml anti E-cadherin Ab (HECD-1; provided by Dr. Takeichi, Kyoto University) was used to induce invasion. After 15 days, the composite gels were fixed with 10% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or subjected to immunohistochemistry. The tumor cells that had become detached from the stratified layer and had migrated into the underlying collagen gel were counted as invading cells at $\times$ 100 magnification using a light microscope. The average number in two representative sections of each culture was taken as the invading cell number. Tumor cells could be discriminated from fibroblasts on the basis of their morphological features including; 1) bigger cytoplasm and nucleus, 2) rounder shape.

Immunoblot analysis Immunoblot analysis was carried out as described previously,[14, 15] with some modification. Briefly, 1×10^6 cells were lysed with 500 μl of extraction buffer (50 m*M* Tris-HCl pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.15 *M* NaCl, 1 m*M* PMSF and 0.1% SDS) for 15 min and centrifuged at 13000 rpm for 20 min. The supernatant was mixed 1 : 1 with a two-fold concentration of sample buffer, 2% SDS and 5% 2-mercaptoethanol. After boiling for 5 min, 15 μl samples were applied to an SDS polyacrylamide gel electrophoresis plate 7.5 (Daiichi Pure Chemical, Tokyo). The fractionated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 h, then incubated with anti c-*Met* polyclonal Ab (Immuno-Biological Laboratories, Fujioka), which recognizes the c-terminal region of the β-subunit (Cys-Val-Asp-Thr-Arg-Pro-Ala-Ser-Phe-Trp-Glu-Thr-Ser-OH), for 1 h at room temperature. Visualization was performed using a Immun-Blot assay kit (Bio-Rad, Hercules, CA).

Assay for HGF concentration The conditioned medium of the last three days of raft culture was collected and centrifuged at 3000 rpm for 15 min. The supernatant was concentrated using an ultrafiltration membrane (cut-off 30000 *d*) 1 : 10 and stored at −20°C until assay. HGF concentration was measured using an enzyme-linked immunosorbent assay kit (Otsuka Assay Laboratories, Tokushima), which is a sandwich method consisting of three steps, as reported previously.[16] The detection limit of this assay is 0.10 ng/ml.

Immunohistochemistry For E-cadherin staining, dewaxed sections were immersed in 0.01 *M* sodium citrate buffer pH 6.0 and heated in a microwave oven for 20 min at 1300 W.[17] These sections were immersed in methanol with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After incubation with 3.0% normal rabbit serum for 30 min, the sections were incubated with 5 μg/ml of HECD-1 at 4°C overnight. They were washed with PBS, then incubated with biotinylated anti-mouse IgG (Vectastain ABC kit, Vector, Burlingame, CA) for 30 min at room temperature, and with ABC reagent (Vectastain ABC kit) for 30 min at room temperature. Positive reaction was visualized with diaminobenzidine supplemented with 0.02% hydrogen peroxide for 5 min and the sections were counterstained with Mayer's hematoxylin.

Cell scattering assay TE2-NS cells were suspended in trypsin-EDTA, and 5×10^3 cells were reseeded into 12-well plates. The cells were cultured in serum-free medium for 3 days, then 10 ng/ml rHGF and/or 30 μg/ml HECD-1 were added to each culture. After a further 24 h incubation, morphological changes were examined using a phase-contrast microscope.

Statistic evaluation The invading cell number and the concentration of HGF were compared by using Student's *t* test. A difference was accepted as statistically significant when the P-value was less than 0.05.

RESULTS

Invasion of tumor cells in raft culture When TE2-NS cell were cultured on type-I collagen gel without fibroblasts, they showed stratified growth on the gel and did not invade the gel (Fig. 1a). The cells did invade the gel when they were cocultured on the gel containing primary fibroblasts (Fig. 1b, 1c). The number of invading cells varied when different lots of fibroblasts were used (Table I, Fig. 2a). Fibroblasts from normal submucosa (PF-N) were more potent inducers of tumor cell invasion than fibroblasts from tumor tissues (PF-T) (617 ± 189 cells vs. 318 ± 138 cells, $P<0.05$). In three out of seven patients (43%), the number of invading cells induced by PF-N was more than 3-fold larger than that by PF-T. Among PF-Ns, PF-5N fibroblasts induced maximum TE2-NS cell invasion (1589 cells), which was 10-fold higher than that induced by PF-7N (119 cells). In this limited number of patients, we found no correlation between invasiveness and clinico-pathologic characteristics (data not shown).

Immunoblot analysis of c-*Met* (Fig. 3) Immunoblot analysis showed that TE2-NS cells expressed c-*Met*/

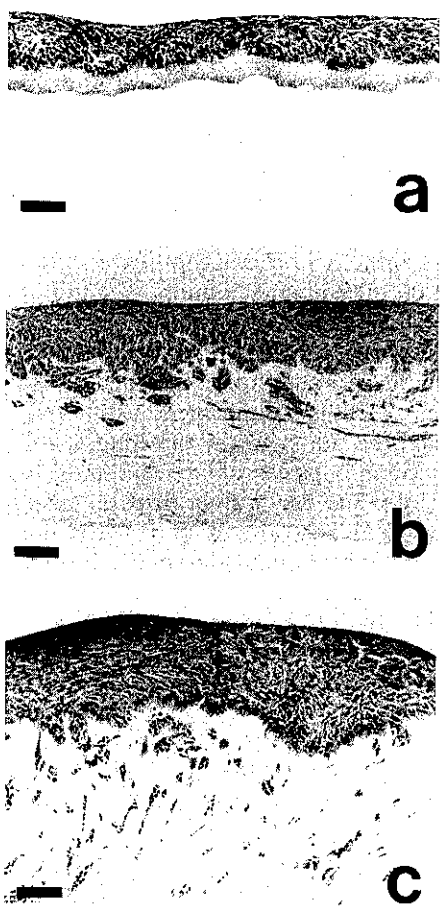

Fig. 1. Invasion assay of TE2-NS cells in organotypic raft culture. TE2-NS cells were cocultured without fibroblasts (a), with fibroblasts derived from tumor tissue (PF-5T) (b), or with fibroblasts derived from normal submucosal tissue (PF-5N) (c). Hematoxylin and eosin staining. Bar = 80 μm.

Table I. Invasiveness of TE2-NS and HGF Concentration in Conditioned Medium of Raft Culture

Patient	PF-T[a]			PF-N[b]		
	Invading cells (*n*)	HGF (+TE2)[c] (ng/ml)	HGF (−TE2)[d] (ng/ml)	Invading cells (*n*)	HGF (+TE2) (ng/ml)	HGF (−TE2) (ng/ml)
Fibroblast free	16	<0.01				
1	29	0.13	nt[e]	475	2.89	nt
2	153	0.07	0.04	736	1.01	1.00
3	782	0.12	0.20	753	4.79	1.13
4	164	0.35	0.25	528	3.86	3.10
5	910	1.83	1.08	1589	5.51	1.45
6	100	0.60	1.21	125	1.19	1.59
7	88	0.69	0.66	119	1.10	1.00
	318 ± 138[f]	0.54 ± 0.23	0.57 ± 0.20	617 ± 189[g]	2.91 ± 0.71[h]	1.54 ± 0.32[g]

a) PF-T, primary fibroblasts from tumor tissue; *b*) PF-N, primary fibroblasts from normal submucosa; *c*), *d*) (+TE2), (−TE2), cocultured with[c] or without[d] TE2-NS; *e*) nt, not tested; *f*) average±SE; *g*) $P<0.05$, *h*) $P<0.01$ vs. PF-T (Student's *t* test).

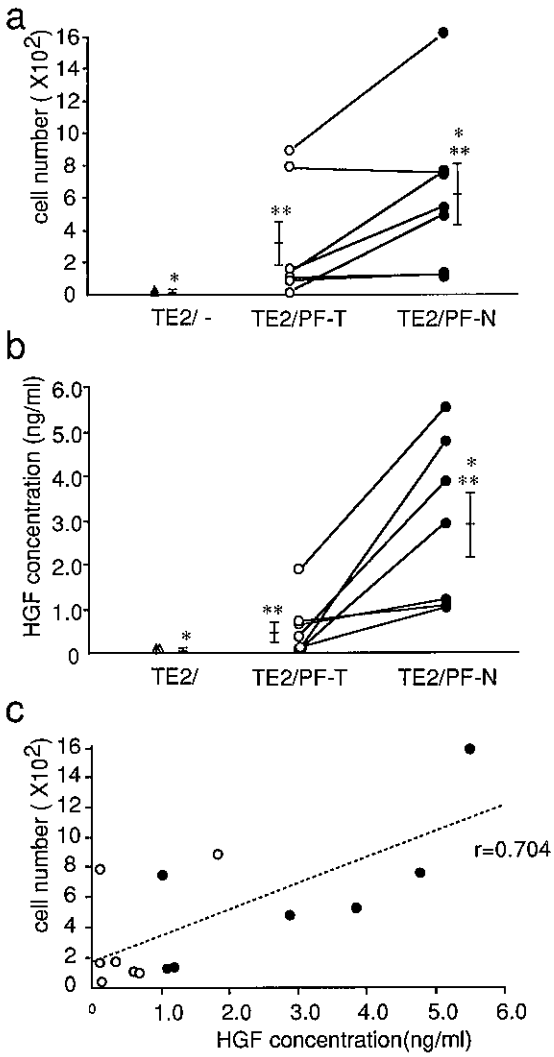

Fig. 2. a, Invasiveness of TE2-NS cells cocultured with or without primary fibroblasts in organotypic raft culture. Cells that had invaded the collagen gel were counted after 15 days of culture. TE2/ - (open triangles), without fibroblasts; TE2/PF-T (open circles), with fibroblasts derived from tumor tissue; TE2/PF-N (closed circles), with fibroblasts derived from normal submucosal tissue. $*P<0.01$. $**P<0.05$ (Student's t test). b, HGF concentration in the conditioned medium for the last 3 days of raft culture. c, Correlation between HGF concentration of conditioned medium and invasiveness of TE2-NS cells cocultured with primary fibroblasts in organotypic raft culture. r: Regression coefficients.

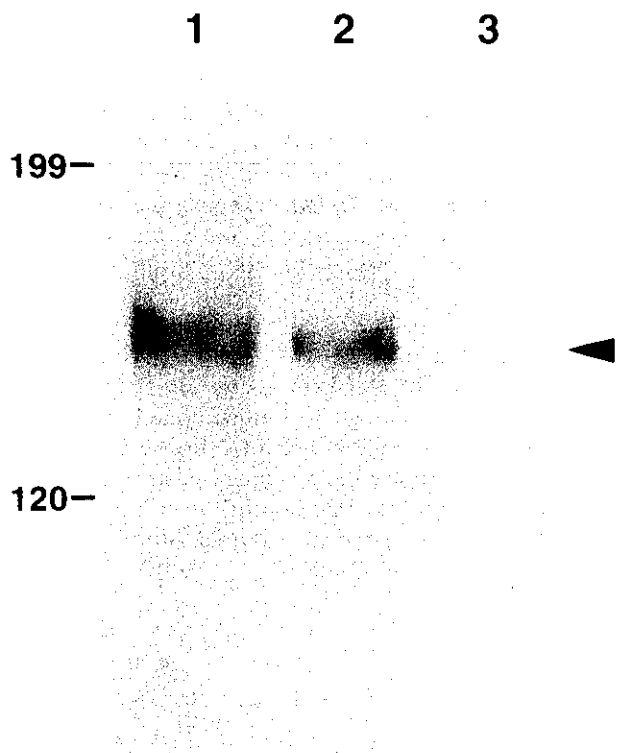

Fig. 3. Immunoblot analysis of c-Met protein. β-subunit = Mr 145000 (arrowhead). Lane 1, A431 as positive control; Lane 2, TE2-NS; Lane 3, primary fibroblasts (PF-5N) as the negative control.

HGF receptor, a proto-oncogene product, at the same level as A431 (positive control). Under reducing conditions, the 145 kD β-chain was detected. No fibroblasts expressed c-Met protein.

HGF concentration in conditioned medium of raft culture (Table I, Fig. 2b) TE2-NS cells did not produce detectable HGF in the conditioned medium when cultured on gel without fibroblasts. Among PF-Ns, PF-5N produced the highest amount of HGF (5.51 ng/ml), which was 5-fold higher than that of PF-2N (1.01 ng/ml). In two out of six normal fibroblasts (PF-3N and PF-5N), the HGF concentration in the case of coculture with TE2-NS was about 4-fold higher than that without tumor cells. This stimulation of HGF production by tumor cells was not recognized with other PF-Ns or any of the PF-Ts. PF-N produced a larger amount of HGF than PF-T with TE2-NS (2.91 ± 0.71 ng/ml vs. 0.54 ± 0.23 ng/ml, $P<0.01$). In four of seven patients (57%), the amount of HGF produced by PF-N was over 10-fold larger than that by PF-T.

The number of invading cells and the HGF concentration of the conditioned medium were well-correlated, as shown in Fig. 2c. The regression coefficient ($r=0.704$) implies that fibroblasts producing larger amounts of HGF induced greater invasion of tumor cells.

Inhibition and induction of tumor cell invasion (Table II) Neutralizing Ab against HGF (10 μg/ml) partially suppressed the invasion of TE2-NS induced by PF-4N fibroblasts, which showed very high HGF production

(Fig. 4b). Most of the tumor cells stayed stratified on the gel and a small number of cells (197 cells) became detached from the stratified layer and migrated into the gel when anti-HGF Ab was used, although a larger number of cells (843 cells) invaded when the same concentration of non-immunized rabbit serum was used (Fig. 4a). Invasion of TE2-NS cells into the collagen gel without fibroblasts was marginally induced at the highest dose (50 ng/ml) of rHGF tested (Fig. 4d). Although marked budding of the stratified layer occurred, only a small number of cells (75 cells) became detached and migrated into the gel.

E-Cadherin expression and cell scattering Since addition of rHGF was not enough to induce complete invasion, we investigated the implication of E-cadherin, an intercellular adhesion molecule, which was also considered to be important for tumor invasion in the raft culture.[7] In immunostaining, E-cadherin was expressed strongly at

Table II. Effects of rHGF and Neutralizing Antibody (Ab) against HGF or Anti E-Cadherin Antibody on Invasion Assay of TE2-NS Cells

Fibroblasts (PF-4N)	Treatment		Invading cells (n)
	Ab	rHGF[a]	
−	−	−	8
−	−	10 ng/ml	36
−	−	50 ng/ml	75
+	−	−	798
+	NRS[b]	−	843
+	HGF Ab[c]	−	197
−	ECD Ab[d]	−	29
−	ECD Ab	50 ng/ml	48

a) rHGF, recombinant HGF; *b*) NRS, nonimmunized rabbit serum; *c*) HGF Ab, 10 μg/ml neutralizing Ab against HGF; *d*) ECD Ab, 30 μg/ml anti E-cadherin Ab (HECD-1).

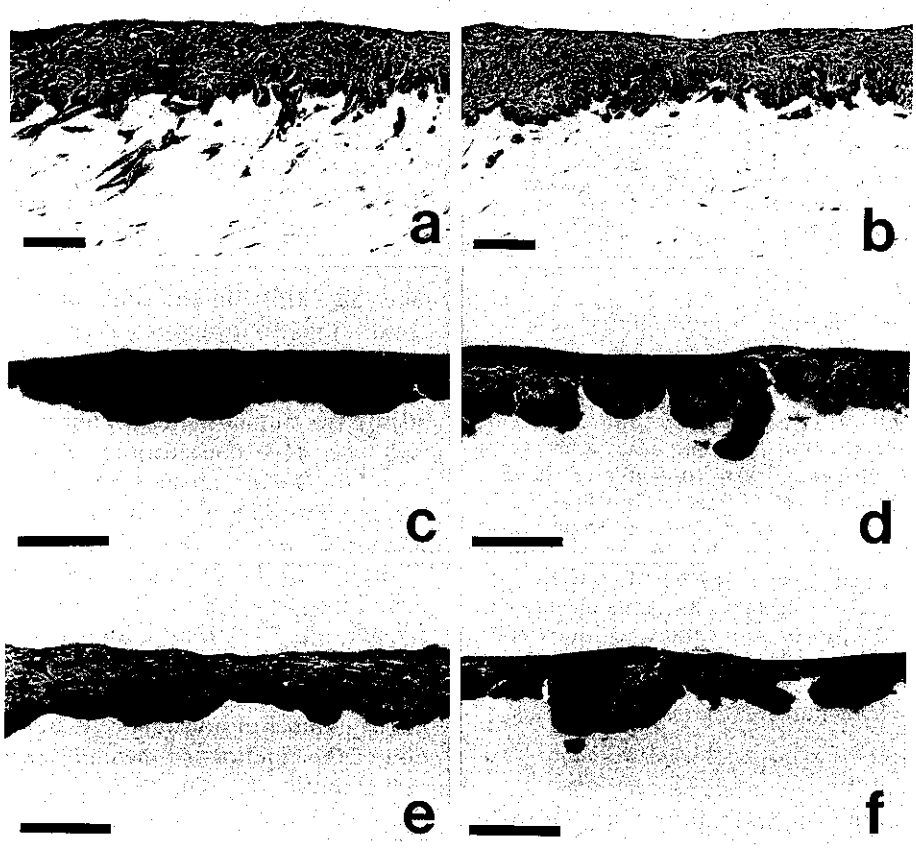

Fig. 4. The effects of rHGF and neutralizing antibody against HGF or anti E-cadherin antibody on invasiveness of TE2-NS cells in organotypic raft culture. TE2-NS cells were cocultured with PF-4N in addition to 10 μg/ml non-immunized rabbit serum (a) or 10 μg/ml neutralizing antibody against HGF (b). TE2-NS cells were cultured on fibroblast-free gel without (c), or with 50 μg/ml rHGF (d), 30 μg /ml anti E-cadherin antibody (e) or both reagents (f). Hematoxylin and eosin staining. Bar = 100 μm.

the cell-cell boundaries of TE2-NS cells stratified on the gel, while the cells that had invaded the collagen gel containing fibroblasts showed reduced expression of E-cadherin, as we had expected (Fig. 5).

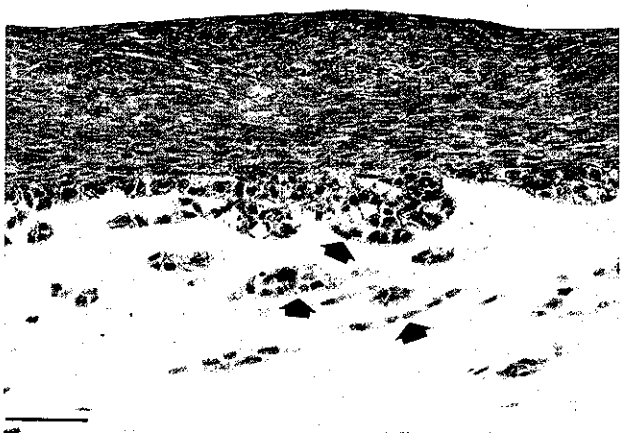

Fig. 5. Immunohistochemical staining of TE2-NS cells cocultured with PF-5N in raft culture using monoclonal antibody against E-cadherin. Invading cells showed reduced E-cadherin expression (arrows). ABC method and hematoxylin counterstain. Bar=30 μm.

Cell scattering on monolayer culture was examined upon addition of rHGF (10 ng/ml) and/or HECD-1 (30 μg/ml). Untreated TE2-NS cells exhibited cobblestone-patterned colonies (Fig. 6a). Either rHGF or HECD-1 was not sufficient to promote cell scattering (Fig. 6b, 6c). However, in both cases, cell-cell adhesion seemed to be loosened, because the edges of the colony were irregular (like budding). Following treatment with both rHGF and HECD-1, they exhibited a dispersion of colony formation and the cell shape changed to a spindle form (Fig. 6d).

In organotypic raft culture, however, invasiveness of TE2-NS cells cultured on the gel without fibroblasts was not induced significantly with 30 mg/ml HECD-1 (29 cells) or even with both 50 μg/ml rHGF and 30 μg/ml HECD-1 (48 cells) (Table II, Fig. 4e, 4f).

DISCUSSION

We used a three-dimensional coculture model in type I collagen gel in order to investigate the mechanism of the invasion of cancer cells induced by fibroblasts. This coculture model has already been employed for the evaluation of invasion ability and for the examination of the invasion mechanism of squamous cell carcinoma.[6, 18] TE2-NS, a cell line derived from squamous cell cancer of

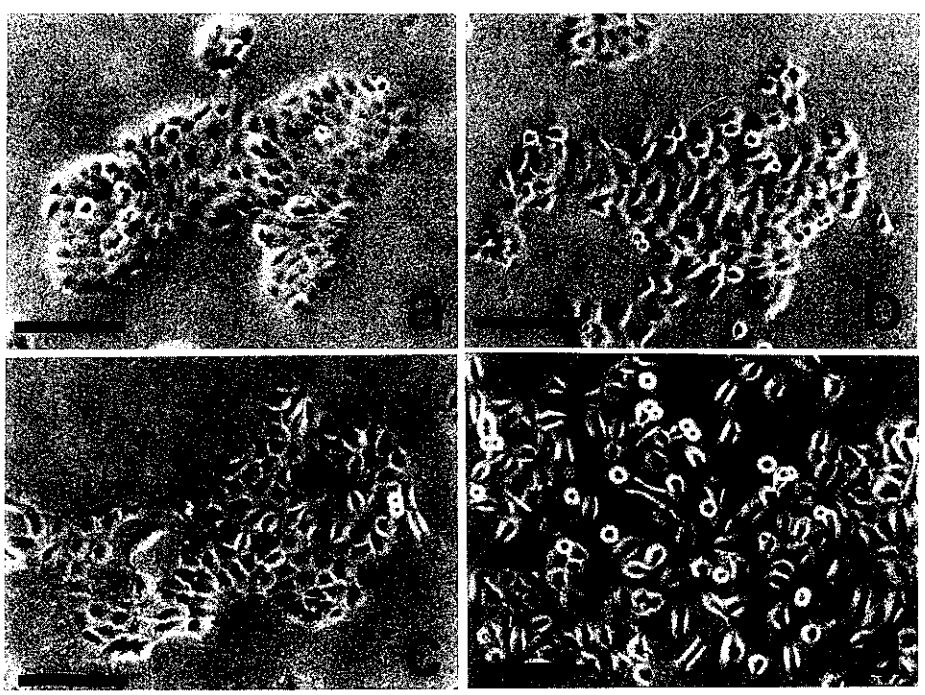

Fig. 6. The effects of rHGF and monoclonal antibody against E-cadherin on scattering of TE2-NS cells in monolayer culture. TE2-NS cells were incubated for 24 h without (a) or with 10 μg/ml of rHGF (b), 30 μg/ml HECD-1 (c) or both reagents (d). Bar=40 μm.

human esophagus, grew stratified on the collagen gel, with strong cell-cell adherence, and showed invasive behavior when cocultured with fibroblasts. The structure was similar to the early phase of human esophageal cancer tissue *in situ*. In this system, various factors, including mutual adhesion of tumor cells, degradation of the matrix adjacent to the tumor cells and migration of tumor cells into the matrix, appear to have influence, as is the case *in vivo*.

HGF, originally identified as a potent mitogen for mature hepatocytes, is a kringle-containing polypeptide growth factor which targets a wide variety of cells.[12, 19, 20] HGF is synthesized by mesenchymal (stromal) cells, including fibroblasts, and enhances the dissociation and motility of epithelial cells; scatter factor was identified as the same molecule as HGF.[21, 22] It has been recently reported that HGF not only induces morphological changes of cells, but also promotes invasiveness and tumor progression.[23] In accordance with this report, immunoreactive HGF concentration in tumor extracts of human breast cancers was found to be the most important independent factor in predicting relapse-free and overall survival.[24] The cellular receptor for HGF has been determined to be c-*met*,[25] a proto-oncogene which is over-expressed in various tumors.[15] Ebert reported that c-Met was overexpressed in pancreatic cancer, in association with increased levels of HGF mRNA.[26]

In the present study, various levels of HGF were produced by primary non-transformed fibroblasts from cancer patients, which induced tumor invasion. HGF concentration in the conditioned medium of raft culture was well correlated to invasiveness of TE2-NS cells. This tumor cell line expressed c-Met protein/HGF receptor but did not produce any detectable amount of HGF, so that it showed only slight invasion in the absence of fibroblasts.

In two out of six patients (PF-3N and -5N), PF-N produced HGF in conditioned medium with tumor cells at about 4-fold higher concentration than without tumor cells. This result suggests that these two fibroblast lines secrete HGF in response to an HGF-inducing factor secreted by TE2-NS cells, as reported in other tumor cells,[27, 28] and that fibroblasts in normal tissue of individual patients have different abilities to produce HGF in response to HGF-inducing factor, and as a consequence, they induce quite different degrees of invasion of the same tumor.

Interestingly, PF-N induced more extensive invasion of tumor cells than PF-T. This difference was consistent with the amount of HGF, because PF-T produced less HGF in the absence of tumor cells, and also none of them responded to the HGF-inducing effect of tumor cells. These results suggest that the local environment of cancer tissue might affect the functions of fibroblasts in normal tissue to produce HGF and to respond to tumor-derived HGF-inducing factor. It was reported previously that fibroblasts derived from breast cancer tissues stimulated the proliferation of breast cancer cells, MCF-7, more than fibroblasts derived from normal breast tissues.[5] In addition to invasion assay as described in this report, we examined their influence on tumor cell proliferation. In our study, there was no difference in proliferation of TE2-NS induced by PF-N and by PF-T (data not shown).

The invasion of TE2-NS induced by fibroblasts was partially inhibited with neutralizing anti-HGF Ab. However, the application of rHGF at high concentration (50 ng/ml) without fibroblasts induced only marginal invasion of TE2-NS, which maintained cell-cell adhesion with strong budding of the tumor cell layer. These results suggest that HGF is an important, but not the sole, paracrine factor inducing the invasion of TE2-NS cells into the collagen gel. Other factors produced by fibroblasts also need to be examined.

Cell-cell adhesion is mainly regulated by homotypic interaction of cadherin molecules,[29] which are anchored to the cytoskeleton via associated cytoplasmic proteins, such as α- and β-catenin.[30] We have reported that the dysfunction of E-cadherin, α-catenin and/or β-catenin is involved in tumoral invasiveness *in vitro* and *in vivo*.[7, 8, 31–33] In the present study, immunostaining of raft culture showed a decline of E-cadherin, and the results of a cell dispersion assay on a monolayer indicated collaboration of rHGF and anti E-cadherin Ab. These results suggest that down-regulation of E-cadherin, as well as stimulation of HGF, is necessary for tumor cell invasion. HGF has been reported to enhance tyrosine phosphorylation of β-catenin and to down-regulate E-cadherin function without affecting protein amount.[34, 35] However, we do not know what fibroblast factor can cause down-regulation of E-cadherin, since rHGF did not induce any quantitative or functional change of E-cadherin immunocytochemistry (data not shown). Moreover, TE2-NS cells still could not readily invade the collagen gel in organotypic raft culture, even when treated with both rHGF and anti-E-cadherin Ab.

The present study strongly suggests the importance of paracrine effects of fibroblasts in humans, partly through HGF secretion. However, even in this well characterized and simplified system, tumor invasiveness appears not to be mediated solely by HGF. Thus, the mechanisms of tumor cell invasion induced by fibroblasts need to be further investigated from many viewpoints, including homotypic tumor cell adhesion, tumor cell proliferation, and secretion of proteolytic enzymes induced by tumor cell-fibroblast interactions.[36]

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