Paracrine effects of hepatocyte growth factor/scatter factor on non-small-cell lung carcinoma cell lines

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Summary We have studied the mitogenic, motogenic and morphogenic effects of hepatocyte growth factor (HGF), also known as scatter factor (SF), on 15 non-small-cell lung carcinoma (NSCLC) cell lines that have had their ras genotype determined. HGF/SF stimulated proliferation in only three cell lines and exerted no mitogenic activity on six lines. The growth of the remaining six lines was inhibited. The mitogenic effects were not related to the ras genotype of these cell lines, but the inhibitory effect was more commonly observed in cell lines with relatively high levels of Met/HGF receptor (HGFR) expression. HGF/SF induced or enhanced both scatter activity on monolayer culture and single-cell invasion in collagen gels in approximately half of these cell lines. Although the ras genotype of tumour cells did not influence the HGF/SF-induced mitogenic activity, cell lines with the mutant ras genotype more commonly demonstrated a spontaneous motogenic activity than those with the wild-type ras genotype. When tumour cells were grown in collagen gels, HGF/SF induced irregular branching extensions of cell aggregates formed by five out of eight adenocarcinoma cell lines, but significant lumen morphogenesis was distinctly absent. The presence of autocrine HGF/SF loop in these tumour cell lines did not influence their spontaneous or HGF/SF-induced mitogenic, motogenic or morphogenic activities. Overall, our data suggest that stimulation of cell motility, rather than proliferation or differentiation, is the predominant paracrine effect of HGF/SF on NSCLC cells in vitro.

Keywords: lung cancer; invasion; metastasis; differentiation

Hepatocyte growth factor (HGF), also known as scatter factor (SF), was originally and independently isolated as a hepatic regeneration factor (Nakamura et al., 1987) and as a fibroblast-derived motogen (Stoker et al., 1987; Weidner et al., 1991). The mature form of HGF/SF is a heterodimer of a 69-kDa α-chain and a 34-kDa β-chain. These are processed by proteolytic cleavage from a single-chain precursor propeptide of 90 kDa. This cleavage is mediated by a serine protease HGF activator and urokinase-type plasminogen activator (Naldini et al., 1992; Miyazawa et al., 1993).

Although HGF/SF is primarily elaborated by mesenchymal cells, its primary effects are on epithelial cells, with activities on cellular proliferation and movement, and on tissue morphogenesis (Weidner et al., 1990; Montesano et al., 1991; Rosen et al., 1994; Matsumoto and Nakamura, 1996). HGF/SF is also a potent angiogenic factor (Bussolino et al., 1992; Grant et al., 1993). Its receptor (HGFR) is the protein product of the c-met proto-oncogene (Met) that encodes a receptor tyrosine kinase (Park et al., 1986; Bottaro et al., 1991). The heterodimeric Met/HGFR consists of an extra-cellular α-chain and a transmembrane β-chain linked by disulfide bonds. Ligand–receptor interaction results in autophosphorylation of tyrosine (Tyr)-1234 and Tyr-1235 residues of the β-chain of Met/HGFR, and an activation of its intrinsic tyrosine kinase activity (Longati et al., 1994; Zhu et al., 1994a). This leads to the autophosphorylation of Tyr-1349 and Tyr-1356 residues, which are essential for the signal transduction of its pleiotropic effects (Ponzetto et al., 1994; Zhu et al., 1994b). These tyrosine residues are essential binding sites for the SH2 domains of p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase), phospholipase C-γ (PLC-γ), pp60c-src, Shc and Grb2 (Graziani et al., 1991; Okano et al., 1993; Ponzetto et al., 1994; Pelicci et al., 1995). Met/HGFR activation also stimulates mitogen-activated protein kinase (MAP kinase)/ERK (Halaban et al., 1992), Ras (Graziani et al., 1993), and p125 focal adhesion kinase (FAK) (Matsumoto et al., 1994). Recent data indicate that binding of the activated Met/HGFR to Grb2 is necessary for the branching tubulogenic effect of HGF/SF on Madin-Darby canine kidney (MDCK) cells (Fournier et al., 1996; Ponzetto et al., 1996), whereas its association with P13-kinase mediates the scattering effect of HGF/SF (Royal and Park, 1995; Ponzetto et al., 1996). In contrast, the mitogenic effect of HGF/SF appears to involve primarily MAP kinase activation and c-fos induction (Nagamine et al., 1996).

Most lung cancer cells express Met/HGFR (Di Renzo et al., 1991; Rygaard et al., 1993), and it is overexpressed in approximately 40% of primary lung adenocarcinoma tissue (Liu and Tsao, 1993a). The expression of HGF/SF by lung cancer cells in vitro and in vivo has also been reported (Yoshinaga et al., 1992; Tsao et al., 1993; Harvey et al., 1996; Olivo et al., 1996). A recent publication also indicated that high levels of HGF/SF protein content in primary non-small-cell lung carcinoma (NSCLC) was correlated with poor prognosis, suggesting that paracrine and/or autocrine
functions of HGF/SF may play important roles in the biology of lung cancer cells (Siegfried et al, 1997). We report here the results of our studies on the mitogenic, morphogenic and morphogenetic activities of recombinant HGF/SF in 15 NSCLC cell lines that have had their ras genotype determined.

MATERIALS AND METHODS

Recombinant human HGF/SF was purified from the conditioned media of mammalian cell lines transfected by an expression vector containing full-length cDNA of human HGF/SF (Montesano et al, 1991; Zionicheck et al, 1995). The stock solutions were stored in aliquots at −70°C. The specific activities of different lots of HGF/SF preparations were checked using scatter assay on MDCK (Madin-Darby Canine Kidney) cells and were found to be similar.

Cell lines and cell culture

The NSCLC cell lines included those previously established in our laboratory (Liu and Tsao, 1993b) and at the NCI-Navy, Bethesda, MD, USA (Phephs et al, 1996). A549 and MDCK cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell lines were cultured in either RPMI-1640 medium plus 10% fetal bovine serum or ACL-4 medium. RPMI-1640 medium and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). ACL-4 serum-free medium was prepared as previously reported (Gazdar and Oie, 1986), but without supplementation with epidermal growth factor (EGF).

HGF/SF/Met protein

The HGF/SF/Met protein levels of the cell lines were determined in cultures that had become confluent. Total cellular proteins were extracted in an aqueous buffer containing 50 mmol l⁻¹ Hepes (pH 8.0), 1% Triton X-100, 10% glycerol, 150 mmol l⁻¹, 10 mmol l⁻¹ EDTA, 100 mmol l⁻¹ sodium fluoride, 10 mm sodium pyrophosphate, 1 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ aprotinin. After centrifugation at 13 000 g for 10 min, the supernatant was collected. Fifty-microgram protein extracts were electrophoretically separated in an 8% sodium dodecyl sulphate (SDS)-polyacrylamide gel, and transferred onto nitrocellulose membrane. After blocking with 5% milk in TBST buffer containing 10 mmol l⁻¹ Tris-HCl (pH 7.0), 0.1% Tween 20, 2.5 mmol l⁻¹ EDTA and 50 mmol l⁻¹ sodium chloride, the membrane was incubated with a polyclonal rabbit antibody against H-Met (catalogue no. SC-161, Santa Cruz Biotech, Santa Cruz, CA, USA), and subsequently revealed using the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim Canada, Dorval, Quebec, Canada).

HGF mRNA expression

Significant levels of HGF mRNA expression were determined using the RNAase protection assay (RPA). An HGF/NK2 cDNA was cloned using the reverse transcriptase polymerase chain reaction (RT-PCR) technique and subcloned into the pGEM-4Z plasmid (Promega, Madison, WN, USA). This cDNA of 446-bp encodes the nucleotides 645–1071 of the alternatively spliced human HGF-NK2 variant mRNA (Miyazawa et al, 1993). The authenticity of this cDNA was confirmed by sequencing, and its orientation established by hybridization to Northern blots of RNA from MRC-5 and human lung fibroblast cell lines (unpublished data). ³²P-labelled antisense HGF cRNA was synthesized from HindIII linearized plasmid using the SP6 RNA polymerase. Total cellular RNA was extracted from cultured tumour cells that have grown to confluence, and 50 μg of RNA was used in each RPA analysis, using the RPAII ribonuclease protection assay kit purchased from Ambion (Austin, TX, USA).

Table 1 The phenotypic and genotypic properties of 15 non-small-cell lung carcinoma cell lines and the mitogenic effect of HGF/SF on them

| Histotype | Cell line | Met/HGFR protein | HGF mRNA expression | Ras genotype (WT/GGT) | HGF effect on proliferation |
|-----------|-----------|------------------|---------------------|------------------------|-----------------------------|
| ADC       | MGH-8     | +++              | −                   | K12 (GAT)              | No effect                   |
|           | MGH-13    | ++               | −                   | WT                     | Stimulation                 |
|           | MGH-24    | +++              | +                   | WT                     | Inhibition                  |
|           | A549      | ++               | ++                  | K12 (AGT)              | Inhibition                  |
|           | RVH-6849  | +++              | −                   | WT                     | Inhibition                  |
|           | NCI-H358  | +++              | −                   | K12 (TGT)              | No effect                   |
| ADSQC     | MGH-30    | +++              | +                   | K12 (TGT)              | Inhibition                  |
|           | NCI-H125  | +++              | −                   | WT                     | Inhibition                  |
| SQCC      | MGH-7     | ++               | ++                  | WT                     | Inhibition                  |
|           | NCI-H157  | +                | −                   | K12 (CGT)              | No effect                   |
|           | NCI-H226  | ++               | ++                  | WT                     | Stimulation                 |
|           | NCI-H520  | 0                | −                   | WT                     | No effect                   |
|           | NCI-H1264 | +                | ++                  | K12 (CGT)              | No effect                   |
| LCUC      | MGH-4     | +                | +                   | WT                     | Stimulation                 |
|           | NCI-661   | 0                | +                   | WT                     | No effect                   |

ADC, adenocarcinoma; ADSQC, adenosquamous cell carcinoma; SQCC, squamous cell carcinoma; LCUC, large cell undifferentiated carcinoma. *Relative expression levels of Met/HGFR were scored based on the result presented in Figure 1: 0, not detectable; +, low; ++, moderate; ++++, high. *Cell lines with wild-type ras gene were designated as WT; cell lines with oncogenic ras genotype were designated by their mutated ras gene: K12, codon 12 of K-ras gene; the parenthesis indicates the sequence of the mutant codon. *Relative levels of HGF/SF mRNA expression are based on the RPA results presented in Figure 2: 0, not detected; +, low; ++, moderate. None of these cell lines express HGF/SF at levels that are similar to the high levels expressed by fibroblast cell lines.
ras genotype

The genotypes of these cell lines for Ha-ras, Ki-ras and N-ras were identified using polymerase chain reaction (PCR) followed by hybridization with allele (mutant)-specific oligonucleotide probes (PCR-ASOH method), as described previously (Tsao et al, 1997).

Cell proliferation

The mitogenic effect of HGF/SF was assayed by the incorporation of [3H]methyl-thymidine (Tdr) into the cellular DNA, as previously reported (Tsao et al, 1993). Briefly, 3000–4000 cells were plated into each well of replicate Falcon’s 12-well tissue culture plates (Becton Dickinson; Bedford, MA, USA). The cells were allowed to enter exponential growth phase 2–4 days after plating, the medium was then replaced with either fresh serum-containing medium (control) or the same medium containing 0.5–10 ng ml−1 of HGF/SF. Three to 4 days later, 1 μCi of [3H]Tdr (67 Ci mole−1; ICN Canada, St Laurent, Quebec, Canada) was added to each well, and cells were further incubated for 20–24 h. They were then thoroughly washed in succession with cold phosphate-buffered saline (PBS), 5% trichloroacetic acid (TCA), and 95% ethanol solution. The plates were then air dried. The DNA was solubilized by an overnight incubation in 0.3 N sodium hydroxide at 37°C, and the radioactivity of incorporated [3H]Tdr was counted using a Beckman LS6000 liquid scintillation counter. For each cell line, the HGF/SF effect was assayed repeatedly for three to five times.

Cell motility on a plastic surface

The scatter effect of HGF/SF on each cell line was recorded during the performance of the mitogenic assay using an inverted phase-contrast microscope (see above). With some lines, cells cultured on 35-mm Falcon tissue culture plates were induced to scatter by HGF/SF, then fixed in a solution containing methanol–acetic acid–dH2O (20:5:75) for 10 min. After rinsing with distilled water, cells were stained with PBS containing 4% Giemsa, then air dried. Microphotographs were taken using an inverted microscope.

Three-dimensional collagen gel culture

Collagen gels were prepared using rat tail type 1 collagen (Becton Dickinson; Bedford, MA, USA). The collagen culture medium was prepared by mixing 10 × RPMI-1640 medium containing the appropriate amount of sodium bicarbonate, fetal bovine serum, 5 mg ml−1 stock solution of type I collagen, and 0.125 N sodium hydroxide in 1:4:4 volume proportions. The collagen medium was aliquoted into 12-well culture plates to form a 1-mm-thick basal layer. After incubation at 37°C for 20 min to harden the gel, the same aliquot of collagen medium containing 1–2.5 × 10⁶ cells was overlaid on the basal layer. After the gels had solidified, liquid medium was added, and the plates were returned to the incubator. After 5–7 days’ culture allowing the suspended cells to form small cysts the medium was replaced by fresh medium with or without 10 ng ml−1 of HGF/SF. Morphological changes were observed and photographed directly using an inverted phase-contrast microscope. After 4 days of

Figure 1 Western blot analysis of Met/HGFR protein levels in NSCLC cell lines. A 50-μg sample of total cellular protein extract from each cell line was separated by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose membranes, and immunoblotted with the antibody to C-terminal peptide of human c-met. The 170-kD precursor (*) and p145 β-chain of Met were detected using the enhanced chemiluminescence reaction. To compare the protein levels of different cell lines, the MGH-8 cell extract was included as a reference in each PAGE analysis. The comparative levels of Met/HGFR protein expressed by these cell lines are summarized in Table 1

Figure 2 The ribonuclease protection assay (RPA) for HGF/SF mRNA expression in NSCLC cell lines. For each RPA reaction, 50 μg of total cellular RNA was used. Using the NK2 CRNA probe (see Materials and methods), the full-length HGF mRNA transcript (HGF-FL) is expected to yield a 301-bp protected fragment, whereas mRNA transcript of the NK2 variant of HGF mRNA is expected to yield a 462-bp protected fragment. The nature of other minor bands is unknown and may represent uncharacterized alternatively spliced HGF/SF mRNA transcripts. The comparative levels of the 301 bp HGF-FL protected fragment are summarized in Table 1
incubation, the gels were harvested and fixed in 10% buffered formalin and embedded in paraffin. Sections were prepared, stained routinely with haematoxylin and eosin and evaluated using light microscopy.

RESULTS

Genotypic and phenotypic characteristics of the NSCLC cell lines

Table 1 summarizes the histological classification of all 15 NSCLC cell lines studied, their ras genotypes and their relative levels of HGFR/Met protein and HGF/SF mRNA expression. Six cell lines had oncogenic activation of their Ki-ras gene. All cell lines except two (NCI-H520 and -H661) expressed variable Met/HGFR protein (Figure 1). The lines that did not show detectable HGFR protein also lacked Met mRNA expression by Northern blot analyses (data not shown). RPA detected HGF/SF mRNA expression in 8 of 15 (53%) cell lines (Figure 2); seven of these also co-expressed HGFR/Met mRNA and protein. The concentrated conditioned media of these HGF/SF-expressing cell lines also induced the scattering of MDCK cells, suggesting the secretion of HGF/SF protein and autocrine loop formation in these cells (Tsao et al., 1993).

Effect of HGF/SF on cell proliferation

Among the fifteen NSCLC cell lines, HGF/SF stimulated the monolayer proliferation of only three lines, MGH-4, -13 and NCI-H226 (Figure 3). The stimulation was concentration dependent, with the maximum effect reached at 2–5 ng 1-1. HGF/SF did not significantly affect the proliferation of six cell lines, but it inhibited the growth of the remaining six lines (MGH-7, -24, -130, A549, RVH-6849 and NCI-H125). This inhibition was also concentration dependent. In a majority of cell lines affected, the maximum effect was observed at 5–10 ng 1-1 HGF/SF.

The mitogenic effect of HGF/SF was not correlated with the histological type of tumour cells (Table 1). Among six adenocarcinoma (ADC) cell lines, HGF/SF stimulated the proliferation of one line (MGH-13), showed no effect in two lines (MGH-8 and NCI-H358) and was inhibitory in three other lines (MGH-24, A549 and RVH-6849). Among five squamous cell carcinoma (SQCC) cell lines, one was stimulated (NCI-H226), one was slightly inhibited (MGH-7) and three were not affected (NCI-H157, -H520 and -H1264). Both adenosquamous carcinoma (ADSOQ) cell lines (MGH-30 and NCI-H125) were inhibited, but the effect on the two large-cell undifferentiated carcinoma (LCUC) cell lines (MGH-4, NCI-H66) was heterogeneous. As expected, the two cell lines that did not express HGFR/Met expression (NCI-H520 and -H661) also were not responsive to the mitogenic effect of HGF. The cell lines that were growth stimulated (MGH-13 and -4, NCI-H226) showed either low or intermediate Met/HGFR levels. In contrast, the majority of cell lines that were inhibited by HGF/SF showed high levels of Met/HGFR expression. Neither the ras genotype nor the presence of a putative HGF/SF autocrine loop appeared to influence the mitogenic responses of these cells to exogenous HGF/SF.

Motogenic and invasion effect of HGF/SF

Cells were assessed for scatter activity in low-density cultures, both spontaneously in routine medium and after treatment with...
Figure 4 A representative microphotographs demonstrating the spontaneous and HGF/SF induced scatter activities in NSCLC cell lines. The NCI-H125 cells (A) normally demonstrated insignificant spontaneous scatter activity, whereas the A549 cells (B) showed a slight degree of spontaneous scatter activity. The treatment with 10 ng ml⁻¹ HGF/SF resulted in marked scatter activities in both cell lines (C H125; D A549). The microphotographs were taken on Giemsa-stained fixed cells, with the bar representing approximately 100 μm.

Table 2 The spontaneous and HGF/SF induced motogenic activity of NSCLC cell lines on a plastic surface and in collagen gels

| Ras genotype | Histotype | Cell line | Scattering (plastic surface) | Invasion (collagen gel) |
|---------------|-----------|-----------|-----------------------------|------------------------|
| Mutant        | ADC       | MGH-8     | +                           | +                      |
|               |           | A549      | +                           | +                      |
|               |           | NCI-H358  | 0                           | NC                     |
| ADSQC         | MGH-30    | 0         | NC                          | +                      |
| SQCC          | NCI-H157  | +         | NC                          | +                      |
|               | NCI-H1264 | +         | NC                          | +                      |
| Wild type     | ADC       | MGH-13    | 0                           | NC                     |
|               |           | MGH-24    | 0                           | +                      |
|               |           | RHV-8849  | +                           | NC                     |
| ADSQC         | NCI-H125  | 0         | NC                          | +                      |
| SQCC          | NCI-H226  | 0         | +                           | NC                     |
|               | NCI-H520  | 0         | +                           | NC                     |
| LCUC          | MGH-4     | 0         | NC                          | NA                     |
|               | NCI-H661  | 0         | NC                          | NT                     |

*When tumour cell lines cultured at colony-forming densities or in collagen gels demonstrated individual cell dispersion, they were scored to show scattering or invasion phenotypes. When these phenomena were noted in the absence of HGF/SF, they were designated as spontaneous activity (+) under the control columns. After treatment with HGF/SF, these activities were either induced (+) or enhanced (↑). If the activities were similar before and after HGF/SF treatment, they were designated as NC (no change). *MGH-4 cell line did not form colonies in collagen gel, NA, not assessed. The cell lines were segregated according to their ras genotypes and histogenesis. SQCC, squamous cell carcinoma; ADSQC, adenosquamous carcinoma; ADC, adenocarcinoma; LCUC, large cell undifferentiated carcinoma.

10 ng ml⁻¹ HGF/SF. Scatter activity was considered absent when tumour cells grew only as tight colonies (Figure 4A), but was considered present when some cells dispersed individually between colonies (Figure 4B–D). When compared with the untreated cells, the HGF/SF-induced scatter activity was scored as either induced, enhanced or unchanged (Table 2). Five cell lines showed varying degrees of spontaneous motility, and it was particularly marked in NCI-H157 and NCI-H1264. Four cell lines with spontaneous scatter activity had a mutation in their Ki-ras gene (Table 2). Only one of the nine cell lines with wild-type ras genes showed spontaneous scatter activity. The difference was statistically significant (Table 3), indicating a correlation between
Table 3 The influence of ras genotype and autocrine HGF/SF loop in NSCLC cell lines on their spontaneous or HGF/SF induced/enhanced cell motility on a plastic surface

| Ras genotype | Absent | Present | P-value |
|--------------|--------|---------|---------|
| Wild type    | 8      | 1       | 0.011   |
| Mutant       | 2      | 4       |         |
| HGF/SF autocrine loop | 4 | 3 | 0.608 |
| Absent       | 6      | 2       |         |

| Ras genotype | Absent | Present | P-value |
|--------------|--------|---------|---------|
| Wild type    | 6      | 3       | 0.622   |
| Mutant       | 3      | 3       |         |

P-values were evaluated using Fisher’s exact test, two-tailed.

oncogenic ras activation and spontaneous motogenic activity in NSCLC cells. In contrast, there was a lack of correlation between the occurrence of spontaneous cellular motility and the presence of a putative HGF/SF autocrine loop (Table 3).

Treatment with 2 ng ml⁻¹ or higher concentrations of HGF/SF induced or enhanced cell scattering in six NSCLC cell lines (MGH-8, A549, RVH-6849, NCI-H125, -H226 and -H358). In two other lines (NCI-H157 and NCI-H1264) that demonstrated the greatest spontaneous motility, further stimulation of scatter activity by HGF/SF could not be evaluated. Both lines with no detectable HGFR/Met levels (NCI-H520 and -H661) also did not show HGF/SF-induced scattering. The ability of HGF/SF to induce or enhance cell motility in these NSCLC cells and the degree of such effects was not influenced by the ras genotype or the levels of Met/HGFR on the cells (Table 3).

The ability of these tumour cells to invade and migrate as single cells inside collagen gels was also evaluated (Figure 5 and Table 2). Four cell lines that demonstrated varying degrees of spontaneous scatter activity (MGH-8, A-549, NCI-H157 and -H1264) in monolayer culture, also showed varying degrees of spontaneous single cell invasion activity in collagen gels. Three other cell lines (NCI-H358, -H226 and -H520) additionally demonstrated a slight degree of such activity. HGF/SF induced or enhanced the collagen gel invasion activity in seven cell lines (MGH-8, A549, NCI-H358, MGH-13, NCI -H125, NCI-H226 and RVH-6849). There was an excellent correlation between the ability of HGF/SF to induce these cells to scatter on a plastic surface and in collagen gels.

Morphogenic effect of HGF/SF

In collagen gels, all SQCC cell lines form irregular cell clusters, whereas most of ADC cell lines formed either random or rod-shaped cell aggregates (Figure 6A), which were composed of solid cords of cells without lumens formation (Figure 5A and C). After the addition of HGF/SF, none of the SQCC cell lines demonstrates morphogenic activity, whereas the cell aggregates of five ADC cell lines (A549, NCI-H358, MGH-30, MGH-13 and RVH-6849)

Figure 5 The ability of NSCLC cells to demonstrate single-cell invasion or locomotion in collagen gels. Cells were seeded in 2.0 mg ml⁻¹ collagen type I gels and allowed to form colonies spontaneously (A and C). After colonies have formed, duplicate wells were treated with 10 ng ml⁻¹ HGF/SF (B and D). After a further 3–5 days’ incubation, the gels were removed from the plates and fixed in 10% buffered formalin solution, embedded in paraffin, and sections were prepared and stained with haematoxylin and eosin. RVH-6849 cells formed tight colonies in control untreated gels (A) but demonstrated single-cell scattering and invasion after treatment with HGF/SF (B). In contrast, NCI-H358 cells demonstrated a slight degree of spontaneous single cells invasion (C), but this activity was markedly enhanced after treatment with HGF/SF (D). The bar lengths represent approximately 50 μm

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showed varying degrees of and irregular branching extensions (Figure 6B). Histological sections, however, failed to show significant lumen formations in these branching cell cords. The ability to show ‘branching extensions’ was not influenced by the ras genotype of these cell lines.

**DISCUSSION**

We have investigated the mitogenic, motogenic and morphogenic properties of fifteen NSCLC cell lines with known ras genotype and their responses to exogenous treatment with recombinant human HGF/SF. The results indicated that HGF/SF may have significant mitogenic and morphogenic effects on these lung cancer cells, but a growth stimulatory effect occurred in only a minority of these cell lines.

Recombinant human HGF/SF exerted heterogeneous mitogenic effects on these cell lines, with six (40%) lines showing no response, and six others (40%) showing an inhibitory effect. Only three cell lines (20%) demonstrated significant growth stimulatory effect. Interestingly, the effect of HGF/SF on the proliferation of lung cancer cell lines has not been extensively reported, and ours represents the most comprehensive study on this subject. Among the four NSCLC cell lines that were studied and reported previously (Tajima et al., 1992; Yoshinaga et al., 1992; Singh-Kaw et al., 1995), HGF/SF stimulated growth in two lines (SQCC and LCUC respectively), and was ineffective in two others. None of these lines was derived from ADC. It is possible that the paucity of reports on the growth stimulatory effect of HGF/SF on lung cancer cells may actually reflect the infrequency of such findings, and this would be consistent with our results. The mitogenic effect of HGF/SF on other human tumour cell lines are very heterogeneous. HGF/SF inhibits the proliferation of all seven human hepatocellular carcinoma cell lines studied (Shiota et al., 1992), demonstrates no significant mitogenic effect on four out of five gastric carcinoma cell lines (Tanafel et al., 1994; Nagamine et al., 1996), and stimulates the growth of three of five malignant melanoma cell lines (Kan et al., 1991; Halaban et al., 1992). In contrast, HGF/SF consistently stimulates the proliferation of normal human epithelial cells in cultures. These include primary cultures of hepatocytes (Nakamura et al., 1987), mammary (Niranjan et al., 1995), biliary (Strain et al., 1995) and bronchial (Tsao et al., 1993) epithelia, keratinocytes (Matsumoto et al., 1991) and melanocytes (Halaban et al., 1992). It is worth noting that the activation of over-expressed tyrosine kinase receptors often leads to a suppression of cell proliferation, independent of the activation of the p21ras and MAP kinase (Osterop et al., 1994). This may explain the inability of constitutive ras activation to block the HGF/SF induced inhibition of proliferation in some of these NSCLC cell lines.

Our results have largely confirmed the prominent motogenic effect of HGF/SF on epithelial cells cultured on plastic surface and in collagen gels, including neoplastic cells. Among thirteen Met/HGFR expressing NSCLC cell lines that we studied, HGF/SF induced or enhanced scattering in six lines. Two other lines already demonstrated high levels of spontaneous cellular motility that precluded the evaluation of further enhancement. There is a very good correlation between the ability of these tumour cells to move on a plastic surface and in collagen gels, suggesting that the primary effect of HGF/SF is on cell movement, regardless of the environment of these cells. Recent reports suggested that the binding and activation of PI3-kinase, but not Grb-2/Sos-Ras pathways is important in HGF/SF-induced cell motility in MDCK cells (Fournier et al., 1996; Ponzetto et al., 1996). This would be consistent with our findings that the ability of HGF/SF to induce motogenic activity is not affected by the constitutive activation of the ras signal transduction pathway. Despite an absence of correlation between the presence of HGF/SF autocrine loop and spontaneous scatter activity, an autocrine motility function of HGF/SF in some of these NSCLC cells cannot be completely excluded. This requires further studies that use techniques to silence the HGF/SF expression and/or function. Recent reports indicated that a significant proportion of primary NSCLC overexpress HGF/SF and Met/HGFR (Harvey et al., 1996; Olivero et al., 1996; Takanami et al., 1996), and high levels of HGF/SF in these tissues may be correlated with a poor overall survival rate (Siegfried et al., 1997). The most important biological process that affects the survival or prognosis of lung cancer patients is metastasis, and cellular motility is one of the critical cellular functions during metastasis.

The observation that oncogenic ras activation is correlated with the spontaneous motogenic activity supports a hypothesis that the ras oncogene may promote early metastasis in NSCLC. Several studies have demonstrated that ras mutations in ADC is a poor prognostic factor, especially for early-stage disease (Rodenhuis and Slebos, 1992; Sugio et al., 1992). These patients usually die of distant metastatic recurrences.

Another important function of HGF/SF-Met/HGFR interaction is in morphogenesis, especially in the formation of three-dimensional tubular/glandular structures (Montesano et al., 1991). This effect has been demonstrated in vitro in normal epithelial cells, including human and mouse mammary epithelial cells (Niranjan et al., 1995),
epithelial cell lines derived from normal human prostate (Brinkmann et al., 1995) and mouse bile ductular and pancreatic epithelial cells (Johnson et al., 1993; Jeffers et al., 1996). In these normal cells, these branching cords of cells demonstrate lumen formations, thus consistent with glandular differentiation. We have also observed HGF/SF induced ‘branching extensions’ when lung ADC cell lines were cultured in collagen gels, but these structures fail to show gland lumen morphogenesis. In contrast, these branching cell cords showed irregular extensions that are more consistent with promotion of cell movement or dispersion. There is actually very scant published evidence to indicate that the glandular morphogenic activity of HGF/SF is functionally and commonly maintained in neoplastic cells. Brinkmann et al (1995) studied the morphogenic activity of HGF/SF in 64 human carcinoma cell lines of various organs and reported significant morphogenic activities in only three of these lines. Among the six lung carcinoma cell lines they studied, HGF/SF demonstrated induction of morphogenic activity in two lines, but only one (LX-1) line showed significant ‘alveolar’-like lumen formation. In fact, only the LX-1 cell line that has been transfected by and is autocrinely expressing HGF/SF showed this effect, which is lost when a neutralizing antibody to HGF/SF was added. This indicates that the parent LX-1 cells do not spontaneously form alveolar structures. The discrepancy between normal and cancer cells in their morphogenic responses to HGF/SF may be caused by post-receptor modification of the Met/HGF receptor signal transduction pathways, or by alteration in the cytoskeletal filament organization in neoplastic cells.

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