Overexpression and activation of hepatocyte growth factor/scatter factor in human non-small-cell lung carcinomas

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Summary Hepatocyte growth factor/scatter factor (HGF/SF) stimulates the invasive growth of epithelial cells *via* the c-*MET* oncogene-encoded receptor. In normal lung, both the receptor and the ligand are detected, and the latter is known to be a mitogenic and a motogenic factor for both cultured bronchial epithelial cells and non-small-cell carcinoma lines. Here, ligand and receptor expression was examined in 42 samples of primary human non-small-cell lung carcinoma of different histotype. Each carcinoma sample was compared with adjacent normal lung tissue. The *Met*/HGF receptor was found to be 2 to 10-fold increased in 25% of carcinoma samples ($P = 0.0113$). The ligand, HGF/SF, was found to be 10 to 100-fold overexpressed in carcinoma samples ($P < 0.0001$). Notably, while HGF/SF was occasionally detectable and found exclusively as a single-chain inactive precursor in normal tissues, it was constantly in the biologically-active heterodimeric form in carcinomas. Immunohistochemical staining showed homogeneous expression of both the receptor and the ligand in carcinoma samples, whereas staining was barely detectable in their normal counterparts. These data show that HGF/SF is overexpressed and consistently activated in non-small-cell lung carcinomas and may contribute to the invasive growth of lung cancer.

Keywords: hepatocyte growth factor/scatter factor; non-small-cell lung cancer; human cancer; *Met* receptor

Hepatocyte growth factor/scatter factor (HGF/SF) stimulates a broad spectrum of epithelial cells (in addition to other selected cell types) to proliferate, move and also carry out complex differentiation programmes, such as morphogenesis and angiogenesis (for reviews see Goldberg and Rosen, 1993). The HGF/SF receptor, encoded by the c-*MET* oncogene (for a review, see Comoglio, 1993), is expressed in several normal human epithelial tissues and is often overexpressed in carcinomas (Di Renzo *et al.*, 1991, 1992, 1994, 1995a, b; Prat *et al.*, 1991).

HGF/SF is expressed in rodent and human lung at a low level (Tashiro *et al.*, 1990; Iyer *et al.*, 1990). However, the lung becomes a major source of HGF/SF, thus behaving as an endocrine organ, producing and secreting HGF/SF in response to distal organ injury (Tashiro *et al.*, 1990; Rubin *et al.*, 1991). The increased level of circulating HGF/SF following partial hepatectomy or chemical injury to the liver correlates to a marked increase of HGF/SF mRNA expression in the intact lung. HGF/SF increases also in regenerating lung after chemically induced lung injury in rat (Yanagita *et al.*, 1992). Experiments *in vitro* show that HGF/SF stimulates mitogenesis and/or motogenesis of human bronchial epithelial cells (Tsao *et al.*, 1993) and alveolar type II cells (Mason *et al.*, 1994). The factor is produced by non-epithelial cells of the lung (Stoker *et al.*, 1987; Rubin *et al.*, 1991) and is considered primarily an endocrine or paracrine mediator. An autocrine activity on cultured human normal bronchial epithelial cells has been reported (Tsao *et al.*, 1993). However, only a small amount of HGF/SF is produced by either lung fibroblasts or bronchial cells, under the same conditions. In addition, HGF/SF is secreted as an inactive precursor (pro-HGF) that binds the extracellular matrix (Mizuno *et al.*, 1992; Naka *et al.*, 1992; Naldini *et al.*, 1992; Masumoto *et al.*, 1991). In the extracellular environment, pro-HGF is converted to the mature heterodimer and

activated either by uPA secreted by the cells themselves (Naldini *et al.*, 1992) or non-specifically by serum proteases, such as blood-coagulation factor XIIa and its homologous protein named HGF-activator (Miyazawa *et al.*, 1993; Shimomura *et al.*, 1995). In human non-small-cell lung cancer, (NSCLC) the levels of uPA and uPA inhibitors (PAI-1 and PAI-2) are significantly altered, the former being higher and the latter lower than in normal tissues (Liu *et al.*, 1995; Nagayama *et al.*, 1994). PAI-2 decrease is significantly related to NSCLC spread to lymph nodes (Nagayama *et al.*, 1994). Bearing this in mind, we postulated that uPA might promote the invasive properties of NSCLC *via* HGF/SF activation. In this paper, we show that HGF/SF is markedly increased in NSCLC in the form of the processed, i.e. biologically active, molecule.

Materials and methods

Tissue samples

Primary carcinomas from 42 patients not previously subjected to chemo- or radiotherapy were analysed. Tissue samples removed at surgery were dissected by the pathologist. Normal and neoplastic tissues were immediately frozen in liquid nitrogen. Tissues were pulverised using a Mikro-Dismembrator (B-Braun) in the presence of liquid nitrogen. Patient characteristics are listed in Table I.

Western blot analysis

Western blot analysis was carried out as previously described (Di Renzo *et al.*, 1991). The powdered whole tissue was dissolved in boiling sodium dodecyl sulphate (SDS) containing buffer, in the presence of the reducing agent β-mercaptoethanol. Equal amounts of proteins (200 μg) were loaded into each lane. Proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Blots were probed with the anti *Met*/HGF receptor monoclonal antibodies or anti-HGF/SF antiserum, and then with HRP-conjugated rabbit anti-mouse immunoglobulins or Protein-A, revealed by ECL (Enhanced

Table I Patient characteristics

	No of patients
Entered	42
Mean age 65 (range 40–76) years	
Sex	
Male	37
Female	5
Histotype	
SCC	23
AC	17
LCUC	2
Stage[a]	
I	23
II	7
IIIA	11
IIIB	0
IV	1
Grading	
G1	1
G2	10
G3	15
Not determined	16
Mean follow-up 12 (range 6–17) months	

[a] Staging according to Mountain (1986). SCC, squamous cell carcinoma; AC, adenocarcinoma; LCUC, large-cell undifferentiated carcinoma.

Chemiluminescence, Amersham, UK). The relative protein expression was quantified by laser densitometric scanning of radiographs.

Anti *Met*/HGF receptor monoclonal antibody (MAb) DQ-13, used for Western blot analysis, was raised against a peptide corresponding to 19 C-terminal amino acids (from Ser[1372] to Ser[1390]) of the c-*MET* human sequence (EMBL Data-Bank accession no. X54559). MAb DL-21 used for Western blot analysis and Mab DO-24 used for immunohistochemistry were directed against the extracellular domain of the *Met* protein (Prat *et al.*, 1991). Anti-HGF/SF H04 antiserum was kindly provided by Dr A Galvani (Pharmacia-UpJohn, Milan, Italy). It is a HGF/SF β-chain-specific antiserum, produced by immunising rabbits with a mixture of synthetic peptides corresponding to sequences within the β-chain of the human factor. MAb against HGF (DV-14) was raised using recombinant HGF/SF secreted by the *Spodoptera frugiperda* insect cells (Sf9), which were infected with the baculovirus vector containing the full size human HGF/SF cDNA, as immunogen (M Prat *et al.*, in preparation).

Recombinant HGF/SF labelling and immunoprecipitation

Recombinant HGF/SF was produced in Sf9 cells, transfected with a baculovirus transfer vector containing the full length HGF/SF cDNA cloned from human liver, as described by Naldini *et al.* (1995). The factor (in its uncleaved precursor form) was purified from tissue culture supernatants by affinity chromatography on heparin–Sepharose column to near homogeneity and labelled with Na[125]I on iodogen-coated polypropylene vials at a specific activity of 13 μCi μg^{-1}. A fraction of the radiolabelled protein was then incubated at 37°C in the presence of fetal calf serum to induce its proteolytic cleavage to the αβ-heterodimeric form. Immunoprecipitation was carried on by incubating both uncleaved and cleaved forms of radioiodinated HGF/SF with affinity purified DV-14 MAb in Tris-buffered saline, sodium azide, 0.2% bovine serum albumin, 0.2% TritonX-100, containing Sepharose protein A, preincubated with rabbit anti-mouse immunoglobulins.

Immunoprecipitation of HGF/SF from tissue samples

For immunoprecipitation, the powdered whole tissue was dissolved in HEPES buffer (25 mM, pH 7.4) containing 10%

glycerol, 150 mM sodium chloride, 5 mM EDTA, 1 mM EGTA, 1% TritonX-100 and protease inhibitors (2 mM phenylmethylsulphonylfluoride, 100 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin, 100 kIU ml^{-1} aprotinin) at 0°C. Extracts were clarified and proteins were immunoprecipitated using anti-HGF MAb. Precipitated proteins were collected and analysed by Western blot as previously described (Di Renzo *et al.*, 1995a).

Histological and immunohistochemical staining

Cryostatic sections (4 μm) were fixed in cold absolute acetone for 10 min and stained with 0.1% toluidine blue in phosphate buffered saline (PBS) for histological examination. Other sections were used for immunoperoxidase (IIP) staining, as previously described (Natali *et al.*, 1990). To avoid false-negative results, from heterogeneous distribution of the epitope, at least three non-consecutive sections of the biopsy were analysed. IIP was performed using the DO-24 and DV-14 MAbs as purified antibodies at concentrations ranging from 10 to 50 μg ml^{-1}. In controls, samples were incubated with an unrelated MAb of the same isotype. For photographic documentation, IIP stain was performed using the ABC Vectastain Elite kit (Mountain View CA USA), according to the manufacturer's instructions. The enzymatic activity was developed using 3-amino-9-ethylcarbazole (AEC) as chromogenic substrate for 8 min. Slides were then rinsed with PBS and counterstained with Mayer's haematoxylin.

Statistical analysis

Statistical analysis of *Met*/HGF receptor and HGF/SF expression in normal and tumour tissue was performed using the Wilcoxon matched-pairs rank test. Correlation of tumour stage and histology with increased expression of either the receptor or the ligand were analysed using the Pearson R and Spearman correlation tests.

Results

Expression of HGF/SF in human NSCLC

HGF/SF expression was examined in fresh samples of non-small-cell lung carcinomas with Western blot analysis. Each carcinoma sample was compared with the unaffected adjacent tissue. Samples were analysed using an antiserum against the HGF/SF β-chain. HGF/SF is secreted as a single chain precursor (pro-HGF) of the approximate M_r of 92 kDa and is cleaved to give the mature heterodimer in the extracellular environment (Naldini *et al.*, 1992). The mature 92 kDa dimer is composed of a 60 kDa α-subunit disulphide-linked to a 32–36 kDa β_1–β_2-subunit. β_1 and β_2 kDa variants differ for the content of carbohydrates (Weidner *et al.*, 1990); α- and β_1–β_2-chains of the mature heterodimer are distinct in gels run in reducing conditions. Specificity of HGF/SF antiserum was demonstrated by blocking HGF/SF binding with the peptides used as immunogen (not shown). Additional experiments showed that HGF/SF antiserum does not recognise the structurally related HGF-like molecules plasmin, thrombin and macrophage stimulating protein (MSP, not shown).

As shown in Figure 1, in most of normal lung tissue samples the antiserum did not detect any pro-HGF nor the 32–36 kDa β_1–β_2-HGF/SF chain. In a few cases (nos 2 and 3, shown in Figure 1), pro-HGF, but not the β-chain of the mature HGF/SF, was observed in normal tissues. Twenty-three out of 42 carcinomas showed a notable increase of HGF/SF ($P < 0.0001$). In all 23 positive cases, the β-chain of the processed HGF/SF, but not pro-HGF, was observed (Figure 1).

Tumours examined were classified according to their biological and clinical features. Table II shows features of tumours overexpressing HGF/SF. NSCLC samples over-expressing HGF/SF belonged to all the histological classes,

representing 50% of the squamous cell and adenocarcinomas. The majority of tumours examined were stage I–II carcinomas (Table I). This did not allow a significative statistical analysis of the correlation between overexpression and disease stage.
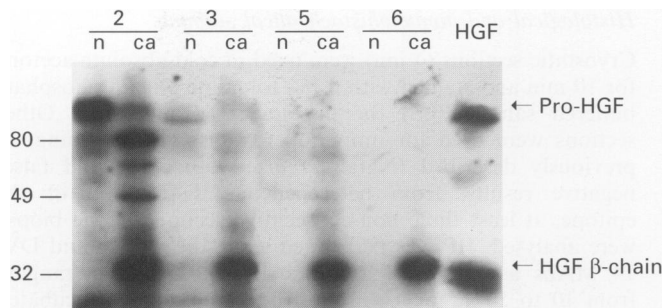
Figure 1 Expression of HGF/SF in human NSCLC samples (ca) compared with samples of normal lung tissue (n) of the same patient, detected with Western blot analysis. Numbers on the top of the lanes indicate different patients. HGF/SF precursor (pro-HGF) and the β-chain of the mature heterodimer were labelled with an antiserum raised against a mixture of synthetic peptides corresponding to sequences within the β-chain of the human factor. Bound antibodies were labelled with HRP-conjugated goat anti-rabbit immunoglobulins and revealed with ECL (Enhanced Chemiluminescence). The supernatant of the Sf9 insect cells, infected with the baculovirus vector containing the full-size human HGF/SF cDNA, was run for a comparison (HGF). Native supernatant and that preincubated with fetal calf serum to get processed HGF were combined. Standards used for M_r estimation reported on the left were prestained bovine serum albumin (80 000), ovalbumin (49 500) and carbonic anhydrase (32 500).

The 36 kDa form of the HGF/SF β-chain was the more prominent in NSCLC, as shown in Figure 1 where it is compared with the HGF/SF $\beta_1-\beta_2$-chain produced by Sf9 insect cells infected with the baculovirus vector containing the full-size human HGF/SF cDNA. As expected, the recombinant HGF/SF $\beta_1-\beta_2$-chain showed a lower M_r because of the different post-translational processing.

To further confirm the identity of the 36 kDa protein, immunoprecipitation experiments with HGF/SF DV-14 MAb were performed by extracting detergent-soluble proteins from powdered whole tissues. Specificity of DV-14 MAb is shown in Figure 2. Additional experiments demonstrated that the HGF/SF MAb does not recognise the structurally related HGF-like molecules plasmin, thrombin and macrophage stimulating protein (MSP, not shown). Proteins immunoprecipitated by MAbs were labelled with anti-HGF/SF antiserum using Western blot analysis. Control experiments were performed with unrelated antibodies. As shown in Figure 3, the 36 kDa protein was precipitated and labelled by anti-HGF/SF antibodies from carcinoma samples and not from the adjacent normal tissues.

Expression of the Met/HGF *receptor in NSCLC*

The expression of the *Met*/HGF receptor was examined in the same samples with Western blot analysis. The receptor is a 190 kDa heterodimeric tyrosine kinase composed of two disulphide-linked chains (Giordano *et al.*, 1989), an extracellular α-chain of 50 kDa and a transmembrane β-chain of 145 kDa. When proteins are separated on SDS-PAGE in the presence of the reducing agent β-mercaptoethanol, the 145 kDa β-chain and the 50 kDa α-chain that constitute the receptor are distinct. In these conditions the 145 kDa β-chain and the p170 precursor were labelled by the DQ-13 MAb against the C-terminal peptide of the human receptor. A representative experiment is shown in Figure 4. The *Met*/HGF receptor of the NSCLC samples was also labelled by

Table II NSCLCs overexpressing HGF/SF and/or *Met*/HGF receptor

No.	Age	Sex	Histology	Grading	TD^a	$Stage^b$	MetR^c	HGF^d
2	59	M	SCC	G3	6.5	I	+	+ + +^{e}
3	62	M	AC	G3	3.0	I	+	+ + +
5	69	M	AC	ND	1.2	I	+ +	+ + +
6	74	F	AC	G3	3.0	I	+ +	+ + +
9	72	M	AC	ND	5.0	IIIA	+ + +	+ +
10	73	M	SCC	G3	6.0	IIIA	+	+ +
12	54	M	LCUC	ND	1.4	I	+ + +	+ + +
13	68	M	SCC	G3	4.0	I	+ +	−
16	76	M	SCC	G1	1.8	I	+ +	+ + +
18	67	M	SCC	ND	6.0	I	+ +	+ + +
19	74	M	SCC	G2	5.5	I	+	+ +
20	68	M	AC	ND	4.5	I	+	+ + +
23	65	F	AC	ND	4.0	II	+	+ +
24	53	M	SCC	G3	8.5	I	+	+ +
25	64	M	SCC	G2	3.0	I	+	+ +
27	70	M	SCC	G2	5.0	I	+ +	−
29	56	F	SCC	G3	5.0	IIIA	+	+ +
30	59	M	AC	ND	5.0	I	+	+ + +
31	72	M	SCC	G3	6.0	IIIA	+	+ +
32	57	M	SCC	G3	5.0	II	+ +	−
34	40	F	AC	ND	8.5	IIIA	+	+ +
35	62	M	SCC	G3	4.0	II	+	+ +
36	67	M	SCC	G2	3.2	II	+	+ + +
40	69	M	SCC	G2	4.5	IV	+	+ +
42	70	M	SCC	G2	6.0	I	+	+ +
43	60	M	AC	ND	7.0	IIIA	+ +	+ +

^{a} TD, tumour diameter (cm). ^{b} Staging according to Mountain (1986). ^{c} Expression of *Met*/HGF receptor (*Met*R) was detected with Western blot analysis; the score relative to normal lung tissue of the same patient was as follows: (−), negative samples; (+), detectable expression as in the normal counterpart; (+ +), 2–5-fold and (+ + +), more than 10-fold increase. ^{d} Expression of HGF was detected with Western blot analysis; the score was: (−), negative samples; (+), detectable expression; (+ +), 2–5-fold and (+ + +), more than 10-fold increase relative to the lowest level of expression detected in carcinomas. ^{e} In this case high level of pro-HGF was found in the normal tissues (see Figure 1). ND, not determined. SCC, squamous cell carcinoma; AC, adenocarcinoma; LCUC, large cell undifferentiated carcinoma.
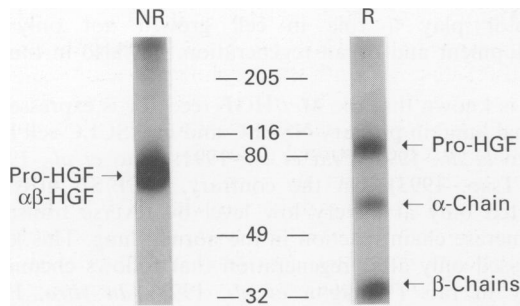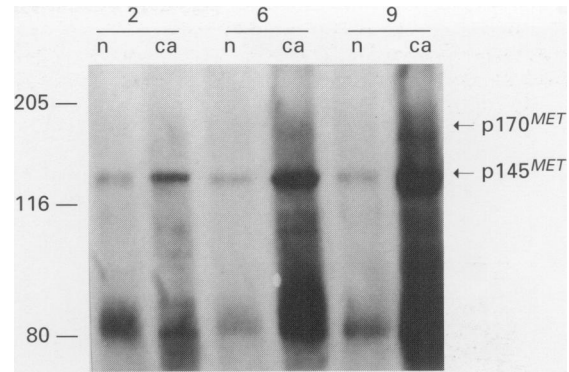
Figure 2 The specificity of anti-HGF/SF DV-14 MAbs is assayed on ^{125}I-labelled HGF/SF, purified from supernatants of Sf9 cells infected with the baculovirus vector containing the full-size human HGF/SF cDNA. When precipitated, HGF was separated in SDS-PAGE analysis in non-reducing conditions (NR); it appears as a single band of about 70 kDa, corresponding to both the mature heterodimeric $\alpha\beta$ complex and the uncleaved HGF/SF precursor (pro-HGF). This band is resolved under reducing conditions (R) in three bands corresponding to pro-HGF (M_r92), the α- and the β-chains of 60 kDa and 32–36 kDa respectively. The two forms of HGF/SF are present, i.e. the uncleaved pro-HGF/SF produced by transfected Sf9 cells and the mature biologically active heterodimer, as precipitation has been performed purposely in the presence of fetal calf serum, which is known to contain a protease able to cleave pro-HGF/SF (Shimomura *et al.*, 1995). Standards used for M_r estimation were prestained myosin (205 000), β-galactosidase (116 500), bovine serum albumin (80 000), ovalbumin (49 500) and carbonic anhydrase (32 500).
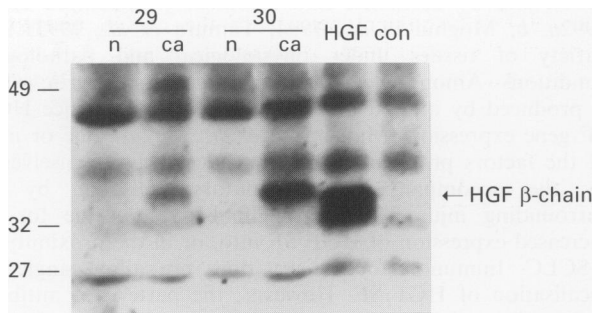
Figure 3 Western blot analysis of HGF/SF immunoprecipitated by anti-HGF/SF DV-14 MAb from human NSCLC samples (ca) compared with samples of normal lung tissue (n) of the same patient. Numbers on the top of the lanes indicate different patients. Western blot analysis with HGF antiserum was carried out as described in the legend to Figure 1. As a control, HGF/SF was also immunoprecipitated from supernatant of the Sf9 insect cells, infected with the baculovirus vector containing the full size human HGF/SF cDNA (HGF), and from control supernatant of non-infected Sf9 cells (con). Standards used for M_r estimation were prestained ovalbumin (49 500), carbonic anhydrase (32 500) and soybean trypsin inhibitor (27 000).

Figure 4 Expression of the *Met*/HGF receptor in NSCLC samples (ca) compared with samples of normal lung tissue (n) of the same patient, detected with Western blot analysis. Numbers on the top of the lanes indicate different patients. The p145 β-chain and the p170 precursor of the *Met*/HGF receptor were labelled by DQ-13 MAbs raised against a synthetic peptide corresponding to the C-terminal tail of the human receptor. Bound antibodies were labelled with HRP-conjugated rabbit anti-mouse immunoglobulins and revealed with ECL (Enhanced Chemiluminescence). Standards used for M_r estimation were prestained myosin (205 000), β-galactosidase (116 500) and bovine serum albumin (80 000).

Adenocarcinomas showed a trend in overexpression of the *Met*/HGF receptor (according to Pearson R test).
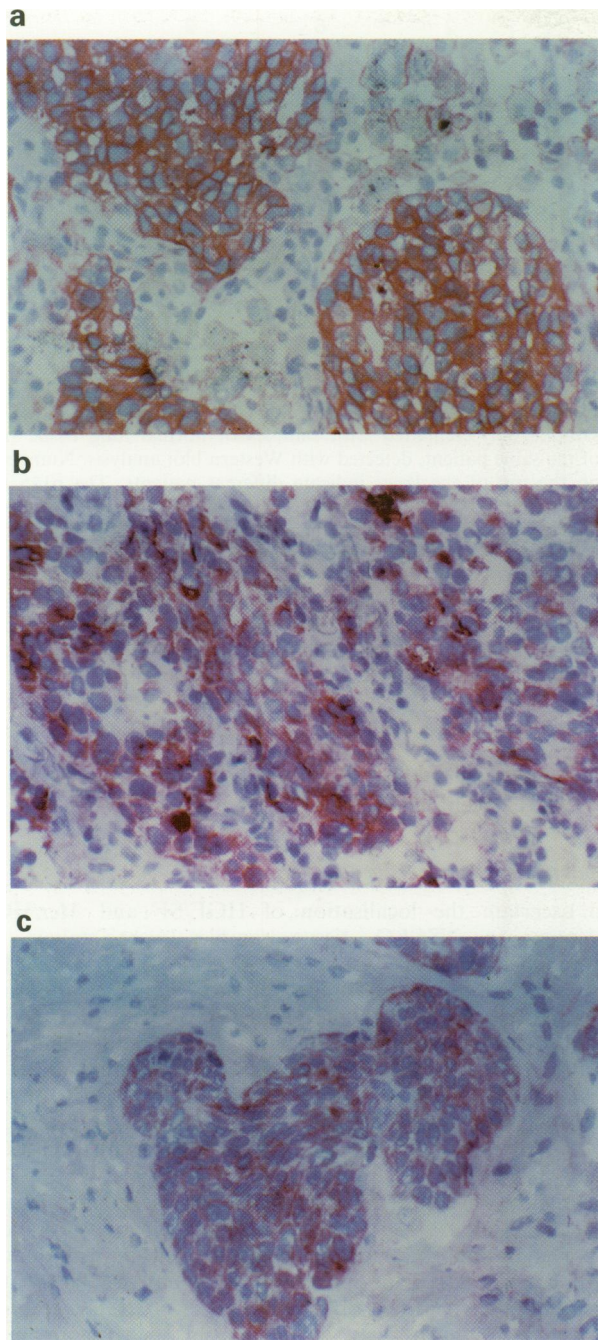
Immunohistochemical localisation

To ascertain the localisation of HGF/SF and *Met*/HGF receptors in NSCLC, tissue samples were stained by immunohistochemistry using the DV-14 and DO-24 Mab respectively (see the Methods section). Staining of normal lung tissues with MAb to the *Met*/HGF receptor (not shown) revealed no immunoreactivity with alveolar walls, lymphoid tissues, cartilage, bronchial glands and vascular walls. A weak staining was observed in the basal aspect of the bronchial epithelium. HGF/SF expression was undetectable in normal tissues by immunohistochemistry with anti-HGF MAb (not shown).

As shown in Figure 5, when comparing carcinoma areas to adjacent normal tissues, a much stronger reactivity for both the ligand and the receptor was detected in carcinomas. Although a cell-specific localisation was not feasible on the basis of the pictures obtained, the *Met*/HGF receptors appeared homogeneously distributed in the tumour mass and localised at the cell surface; whereas the ligand was detected in the cytoplasm of single or grouped cells, scattered in the tumour sample.

Discussion

Several reports describe an increased expression of growth factors and growth factor receptors in human tumours. In many instances, paracrine and autocrine loops have been implicated in growth control of cancer cells. However, the contribution of each growth factor and receptor to the pathogenesis of human cancer is debatable. It is reasonable to speculate that the interplay between different factors and receptors, rather than a single element, play a major role in determining cell proliferation, cell death or both. Here we report that HGF/SF is overexpressed in NSCLC. Data presented here are interesting because of: (1) the HGF/SF property to stimulate not only mitogenesis, but also cell motility and invasiveness; (2) the fact that both the factor and the receptor, encoded by the c-*MET* oncogene, are present in tumour samples; (3) the fact that both the receptor and the ligand are overexpressed in a significant percentage

DL-21 MAb directed against the extracellular domain of the receptor (not shown). The receptor was detectable in most normal lung and carcinoma samples. No gross structural alterations of the protein were ever detected in NSCLC. In 11 out of 42 carcinomas the expression was increased 2 to 10-fold relative to normal tissues ($P=0.0113$; Table II). Increased levels of receptor expression were found in approximately 25% of both squamous cell (6/23) and adenocarcinomas (4/17) and in one of the two large-cell undifferentiated carcinomas. The panel shown in Figure 4 includes squamous cell carcinomas (case no. 2) and adenocarcinomas (cases no. 6 and 9). Statistical analysis of the correlation between overexpression and disease stage and overexpression and tumour histology was performed.

Figure 5 Indirect ABC immunoperoxidase detection of the Met/ HGF receptor (a) and HGF/SF (b and c) on cryostatic sections of NSCLC. a and b are sections of the same clinical case (Grade 2 adenocarcinoma). The receptor, detected with the DO-24 anti-Met MAb, is homogeneously expressed in adenocarcinoma cells (a) whereas HGF expression, detected with the DV-14 anti-HGF MAb, is heterogeneously distributed in the cytoplasm of scattered cells in both adenocarcinoma (b) and in a Grade 3 squamous cell carcinoma (c). (a, ×200; b and c, ×120 original magnification).

of NSCLCs compared with expression in normal tissue of the same patients; (4) the marked level of HGF/SF overexpression; and, in particular, (5) the presence of HGF/SF in its biologically active form in NSCLC.

HGF/SF is a cytokine with unique properties (exerting multiple functions), such as mitogenic and motogenic effects on a variety of normal and transformed cells, and angiogenic (Bussolino et al., 1992; Grant et al., 1993) and morphogenic activity (Montesano et al., 1991). These diverse activities are mediated via a single receptor encoded by the c-MET protooncogene (Naldini et al., 1991; Weidner et al., 1993), overexpressed and/or activated in transformed cells (Cooper et al., 1984; Di Renzo et al., 1991; Giordano et al., 1989;

Park et al., 1986). Hence, it is believed that HGF/SF and its receptor play a role in cell growth not only during development and organ regeneration, but also in tumorigenesis.

It is known that the Met/HGF receptor is expressed in the normal lung, in primary NSCLC and in NSCLC cell lines (Di Renzo et al., 1991; Prat et al., 1991; Tsao et al., 1993; Liu and Tsao, 1993). On the contrary, HGF/SF mRNA was detected only at a very low level by reverse transcriptase-polymerase chain reaction in the normal lung. This level was increased only after regeneration that follows chemical lung injury in rats (Yanagita et al., 1993). In vitro, HGF/SF stimulates mitogenesis and/or motogenesis of bronchial epithelial cells (Tsao et al., 1993) and alveolar type II cells (Mason et al., 1994). Here, we show that both HGF/SF and the Met/HGF receptor are co-expressed (and overexpressed at a significant percentage) in clinical samples of NSCLC, suggesting that ligand and receptor might interact via either a paracrine or autocrine mechanism. Both these mechanisms were hypothesised on the basis of in vitro experiments in cultured cells (Naldini et al., 1991; Liu and Tsao, 1993; Tsao et al., 1993). Whatever the action mechanism might be, overexpression supports the idea that a local activation of the biological activities mediated by the Met/HGF receptor occurs in vivo.

HGF/SF, in particular, is expressed at a considerably high level in NSCLC; bearing in mind that in normal lung, in other organs and also in cell lines, the protein is often barely detectable and the corresponding mRNA is present at very low levels. HGF/SF expression is controlled primarily at transcription level and is cell type-specific (Liu et al., 1994; Planschke-Schutter et al., 1995). It is regulated during development (DeFrances et al., 1992; Sonnenberg et al., 1993), and by hormones and cytokines (Matsumoto et al., 1992a, b; Moghul et al., 1994; Tamura et al., 1993) in a variety of tissues under physiological and pathological conditions. Among the regulatory molecules, injurin, which is produced by injured organs, is known to influence HGF/ SF gene expression (Matsumoto et al., 1992a). One or more of the factors produced either by cancer cells themselves or by the accompanying inflammatory cells or by the surrounding injured tissues might be responsible for the increased expression of HGF/SF into, or in the proximity of, NSCLC. Immunohistochemistry does not allow single cell localisation of HGF/SF. However, the pattern of antibody staining in serial section of the same tumours suggests that stromal cells or inflammatory cells rather than the epithelial tumour cells are responsible for HGF production.

HGF/SF was overexpressed and invariably found in the cleaved biologically active form in 50% of NSCLCs. By contrast, in the corresponding normal tissues, HGF/SF was occasionally detected and was exclusively in the inactive single chain pro-HGF form. It has been shown that native HGF/SF is secreted by cells as pro-HGF. Activation takes place by proteolytic cleavage of pro-HGF at the Arg_{494}– Val_{495} bond. Processing to the mature heterodimer is necessary for HGF/SF to become competent in activating the Met/HGF receptor and to induce biological responses in target cells. Three converting enzymes have been described so far in serum and tissues (Naldini et al., 1992; Mars et al., 1993; Miyazawa et al., 1993; Shimomura et al., 1995); these are serum proteases, such as blood coagulation factor XIIa and its homologous protein named HGF-activator, and the urokinase-type plasminogen activator (uPA). Of these three, serum proteases might bring about quantitative activation of pro-HGF when the blood coagulation cascade is triggered, for example after tissue injury (Miyazawa et al., 1994). However, new results have shown that HGF/SF produced and secreted after partial hepatectomy or unilateral nephrectomy remains as an inactive single chain (Tang et al., 1995). On the other hand, uPA which is increased at the surface of cancer cells, may trigger a more specific local activation of pro-HGF on membrane of target cells and in tissue microenvironment (Naldini et al., 1995). In tissue

microenvironment, macrophages, which are the most efficient activators of pro-HGF (Naldini et al., 1995), may play an additional role. A correlation between uPA activity and tumour cell invasion and metastasis has been well documented. It is worth noting that a correlation between the expression level of both uPA and uPA inhibitors and the onset and progression of human NSCLC has been reported (Liu et al., 1995; Nagayama et al., 1994). The enhanced expression of uPA or the reduction of uPA inhibitors might result in the activation of pro-HGF and in the stimulation of a circuit to induce growth and invasion through the Met/HGF receptor.

In vitro, carcinogenesis may be achieved via a 'single hit' event. It is known that the transformed phenotype is attained after single high-level overexpression of the viral ras or after an autocrine loop is established. Similarly, in vitro overexpression of many growth factor receptors results in ligand-dependent transformation. In these cases, a quantitative effect may be envisaged. In vivo, carcinogenesis is usually a complex multi-step process, involving deletion of tumour-suppressor genes and activation of dominant oncogenes. In NSCLC, the accumulation of ras mutation, p53 mutation and over-expression of growth factors and growth factor receptors have been reported and were associated with the malignant phenotype of tumour cells and/or to patient survival (Ankrapp and Bevan, 1993; Carbone et al., 1994; Liu and Tsao, 1993; Rosell et al., 1993; Yu et al., 1994). Here, we report that the expression of HGF/SF is markedly increased in NSCLC and, more notably, HGF/SF is activated in cancer samples. Several reports implicated HGF/SF in tumour progression and metastasis. Met/HGF receptor activation induces cell motility (Stoker et al., 1987), invasion of collagen matrices (Weidner et al., 1993), activation and secretion of proteases (Rong et al., 1994) and angiogenesis (Bussolino et al., 1992; Grant et al., 1993). The expression of a functional c-MET-encoded receptor is sufficient to transfer an invasive phenotype to transfected cells in the presence of HGF (Giordano et al., 1993). The creation of an HGF autocrine loop either in fibroblasts or in epithelial cells induces invasive properties in vitro and metastatic ability in vivo (Rong et al., 1994; Bellusci et al., 1994). Liver metastases of human colorectal cancer show overexpression and amplification of the c-MET oncogene (Di Renzo et al., 1995b). In conclusion, the overall data suggest that HGF/SF overexpression and activation may be the driving factor for NSCLC cell invasive phenotype acquisition.

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