A scatter factor-like factor is produced by a metastatic variant of a rat bladder carcinoma cell line

Savério Belluscio*, Ginette Moens, Jean Paul Thiery and Jacqueline Jouanneau

Laboratoire de Physiopathologie du Développement, CNRS, URA 1337 and Ecole Normale Supérieure, 46, rue d’Ulm, 75230 Paris Cedex 05, France

*Author for correspondence

SUMMARY

The rat bladder carcinoma epithelial NBT-II cell line undergoes, in vitro, a morphological transition to a fibroblast-like state in the presence of different growth factors. We have selected, in vivo, a metastatic clone, designated M-NBT-II, which has a mesenchymal phenotype and secretes into the culture medium a factor able to dissociate epithelial clusters of NBT-II or MDCK cells. This factor was designated scatter factor-like (SFL) by analogy to the HGF/SF, which has the same dissociating effect in these two cell lines. Here, we show that SFL factor and HGF/SF are different factors: (i) no HGF/SF transcripts could be detected using either specific rat HGF/SF cDNA probes or PCR; (ii) blocking antibodies against rat HGF/SF do not inhibit the SFL activity; and (iii) crude culture medium or partially purified SFL factor-containing fractions do not induce MDCK tubulogenesis, a biological assay that is specific for HGF/SF activity in vitro. We report the partial purification of the SFL factor, based on ion exchange and reverse-phase chromatography. The results indicate that the M-NBT-II metastatic variant secretes a dissociating factor sharing some common biological properties with the HGF/SF, which suggests that the SFL factor is a member of the HGF/SF family and may be involved in tumor progression.

Key words: epithelial cell, scatter activity, metastasis, HGF/SF, SFL

INTRODUCTION

The appearance of invasive forms of carcinoma that can metastasize to distant sites is associated with a poor prognosis. The dissemination of potentially metastatic tumor cells from the primary lesion depends in part on modulation of the cell-cell adhesion and on interaction with the surrounding extracellular matrix (Dorud and Hart, 1993). Growth/scatter factors may be directly involved in the dissociation and in the acquisition of motility by the malignant cells through an autocrine or a paracrine mechanism.

Several molecules that induce cell motility have now been described. The autocrine motility factor (AMF), a 55 kDa protein isolated from conditioned medium of human melanoma cells, enhances the motility of the melanoma cells from which it is secreted (Liotta et al., 1986a). Other AMF-related factors have also been described (Evans et al., 1991; Liotta et al., 1986b). The migration-stimulating factor (MSF), another autocrine factor, is a 70 kDa protein produced by fetal but not adult fibroblasts and secreted by fibroblasts of patients with breast cancer (Grey et al., 1988; Schor et al., 1988).

Molecules that induce cell motility have also been described as growth factors. Insulin-like growth factor II has been reported to act as an autocrine mitogen and a motogen for human rhabdomyosarcoma cells in vitro (El-Badry et al., 1990). IGF-I and II are chemotactic agents for human A2058 melanoma cells (Schiffmann, 1990; Stracke et al., 1989). The epidermal growth factor (EGF) modulates the epithelial phenotype of human A431 carcinoma cells by inducing morphological changes such as membrane ruffling and extension of protrusions (Chinkers et al., 1979).

The hepatocyte growth factor/scatter factor (HGF/SF), both a growth and a dissociating factor, is a glycosylated disulfide-linked heterodimer consisting of a 62 kDa α-chain and a 34/32 kDa β-chain. Functional studies have demonstrated that cleavage is required for biological activity and that the first two out of the four kringle domains, of the α-chain, are necessary for the scatter activity, whereas a complete mature HGF/SF protein seems to be needed for the mitogenic activity (Rosen et al., 1990; Adams et al., 1991; Hartmann et al., 1992; Lokker et al., 1992). The β-chain exhibits a strong homology with the catalytic domain of serine proteases but has no enzymatic activity, since the catalytic site is different (Miyazawa et al., 1989; Nakamura et al., 1989; Matsumoto and Nakamura, 1992). HGF/SF has been described as a fibroblast-derived paracrine multifunctional factor that induces hepatocyte proliferation (Matsumoto and Nakamura, 1992) and that scatters epithelial cell clusters (Gherardi et al., 1989; Hartmann et al., 1992; Lokker et al., 1992). HGF/SF has been described as a fibroblast-derived paracrine multifunctional factor that induces hepatocyte proliferation (Matsumoto and Nakamura, 1992) and that scatters epithelial cell clusters (Gherardi et al., 1989; Hartmann et al., 1992; Lokker et al., 1992). HGF/SF has been described as a fibroblast-derived paracrine multifunctional factor that induces hepatocyte proliferation (Matsumoto and Nakamura, 1992) and that scatters epithelial cell clusters (Gherardi et al., 1989; Hartmann et al., 1992; Lokker et al., 1992). HGF/SF has been described as a fibroblast-derived paracrine multifunctional factor that induces hepatocyte proliferation (Matsumoto and Nakamura, 1992) and that scatters epithelial cell clusters (Gherardi et al., 1989; Hartmann et al., 1992; Lokker et al., 1992). HGF/SF has been described as a fibroblast-derived paracrine multifunctional factor that induces hepatocyte proliferation (Matsumoto and Nakamura, 1992) and that scatters epithelial cell clusters (Gherardi et al., 1989; Hartmann et al., 1992; Lokker et al., 1992).
(Adams et al., 1991), normal bronchial human epithelial and lung carcinoma cells (Tsao et al., 1993), may act as autocrine factors.

We have shown previously that exogenously added transforming growth factor α, acidic fibroblast growth factor (aFGF) and HGF/SF induce the dissociation and the motility of bladder NBT-II carcinoma cells in vitro (Gavrilovic et al., 1990; Vallés et al., 1990; Belluscì et al., 1994). When these factors are constitutively expressed and secreted by NBT-II cells, transfected with the appropriate cDNA, the invasiveness of these cells, in vitro, is enhanced (Gavrilovic et al., 1990; Jouanneau et al., 1991, Tucker et al., 1991) as well as their metastatic behavior in vivo (Jouanneau et al., 1994; Jouanneau and Thiery, unpublished results; Belluscì et al., 1994). These results suggest that tumor cells, which could become autocrine for such growth/scatter factors, would acquire a more advanced stage in tumor progression.

Although these factors act also as motility factors, they seem to enhance the progression of the NBT-II carcinoma. Studies for identifying motility factors naturally produced in vivo within the tumor have not been reported so far. In this work, we have investigated whether an endogeneous growth/scatter factor could be naturally produced by a subset of cells during tumor progression. We have selected, after two passages in mice, a metastatic variant of NBT-II epithelial carcinoma cells that exhibits a stable fibroblastic phenotype. This metastatic variant was named M-NBT-II. Unlike the parental epithelial NBT-II cell line, it produces and secretes a scatter activity that dissociates epithelial NBT-II cells as well as MDCK cells. In this report, we describe this autocrine scatter activity due to a novel factor, termed the scatter factor-like (SFL) factor. Although the SFL factor shares similar biological effects with HGF/SF, we demonstrate that SFL factor and HGF/SF are nevertheless different.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies against rat HGF/SF described previously (Montesano et al., 1991b; Belluscì et al., 1994) and human and rat recombinant HGF/SF (hrHGF/SF and rrHGF/SF) were provided by Prof. T. Nakamura (Osaka University Medical School, Osaka, Japan). Murine monoclonal antibodies against desmplakinins were a gift from Prof. W. Franke (German Cancer Research Center, Heidelberg, Germany). These antibodies allowed us to quantify the cell-cell desmosomal junctions and have been routinely used to determine the dissociating activity of growth factors, leading to the EMT (epithelial-mesenchymal transition) of NBT-II cells (Boyer et al., 1989; Vallés et al., 1990; Jouanneau et al., 1991).

Cell culture

A stable epithelial cell line (E-NBT-II) was subcloned, in vitro, from the NBT-II carcinoma line (Toyoshima et al., 1971) and the metastatic NBT-II cell line (M-NBT-II) was cloned from a nude mouse lymph node metastasis (see below). The normal human lung fibroblast MRC5 cell line (obtained from Dr W. Birchmeier, Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany) and the NIH 3T3 ras D4 cell line (obtained from Dr E. Gherardi, University of Cambridge, Cambridge, England) were used as a source of HGF/SF (Stoker et al., 1987). MDCK cells were obtained from Dr Birchmeier. All these cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum and antibiotics (standard medium). Cell culture, in 3-D collagen gels, was carried out as reported previously (Montesano et al., 1991a,b).

Selection of a metastatic NBT-II variant (M-NBT-II)

For each experiment, six-week-old female Swiss nude mice were subcutaneously injected in the flank with 3.5×10⁶ E-NBT-II cells suspended in 0.1 ml PBS. At autopsy, animals were carefully examined for metastatic foci. Micrometastases were detected after in vitro culture of the lymph nodes as reported previously (Jouanneau et al., 1994). The metastatic and mesenchymal NBT-II clone, called M-NBT-II, was selected, in vivo, after two cycles of subcutaneous injection into the nude mouse. E-NBT-II cells were injected into nude mice and a lymph node micrometastasis was first obtained. These cells, morphologically homogeneous in vitro, called E/M-NBT-II (EM for epithelio-mesenchymal), were injected in a second series of tumor induction in nude mice. One resulting lymph node micrometastasis gave rise to the metastatic variant M-NBT-II (see Fig. 1).

Scatter activity assay

MDCK and E-NBT-II cells were cultured in 6-well plates and incubated with either serum-free M-NBT-II-conditioned medium (SFL-containing medium) complemented with 10% FCS, or with control medium containing recombinant HGF/SF. Cells were examined by phase-contrast microscopy after either 24 hours (MDCK cells) or 48 hours (NBT-II cells). The SFL biological activity was monitored and quantified on MDCK cells as previously described for HGF/SF (Weidner et al., 1990). Briefly, MDCK cells were cultured on a 96-well plate (4000 cells per well) together with serial dilutions, using standard medium, of each fraction to be tested. The scattering effect on MDCK cells was monitored by light microscopy after an overnight incubation. One unit of SFL activity was defined as the lowest amount of scattering activity per ml that clearly dissociates MDCK cells.

Morphogenetic assay

The tubulogenic activity was assayed as described for HGF/SF (Montesano et al., 1991b). MDCK cells were cultured in collagen gels for one week and Bio Rex 70 partially purified SFL or SFL-containing media were added. Recombinant HGF/SF, heparin-Sepharose-purified factor or HGF/SF-containing media were used as positive controls.

Inhibition of HGF/SF activity

HGF/SF scattering activity was inhibited by polyclonal antibodies against rat HGF/SF at a final concentration between 1 and 5 μg/ml IgG. Rat recombinant HGF/SF (10 ng/ml) or serum-free NIH 3T3 ras D4 conditioned medium, containing mouse HGF/SF (6 units/ml), was incubated for 3 hours at 37°C with the antibodies prior to use. The same experiments were carried out with SFL-containing medium (6 units/ml) or Bio Rex 70 and heparin-Sepharose partially purified fraction (4 units/ml).

Purification of the SFL

M-NBT-II rat bladder carcinoma cells were grown to subconfluency in cell culture flasks (250 flasks of 160 cm² each batch, Costar Data Packaging Corp., Cambridge, MA), washed four times with PBS, and further cultured in serum-free medium (20 ml per flask) for 1 day. The conditioned medium was incubated with the Bio Rex 70 resin and the SFL factor was eluted, with a 60 ml linear gradient from 0 to 1.0 M NaCl in the same buffer, at 1 ml/min. The SFL containing fractions were pooled and further purification was performed either by anion exchange chromatography, on a Mono Q column, or by reverse-phase chromatography, on a Nucleosil C 18 column (RP-HPLC). (For details see Fig. 4.) At all steps, the biological activity of the fractions was monitored on MDCK cells and samples were analyzed by SDSPAGE under reducing conditions (Laemmli, 1970), followed by silver
staining (Wray et al., 1981). Protein quantification was carried out with the Bradford reagent (Bio-Rad, Laboratories GmbH, München, Germany).

Preliminary experiments have shown that SFL factor binds to heparin and a purification based on this affinity was attempted. A 500 ml sample of clarified and pH-stabilized M-NBT-II supernatant was incubated overnight, under agitation at 4°C, with 8 ml of heparin-Sepharose beads equilibrated in 50 mM Tris-HCl, pH 7.5. Heparin-Sepharose was then packed into an 8 ml column and connected to an FPLC system; the column was washed with 50 mM Tris-HCl, pH 7.5, and then with the same buffer containing 0.4 M NaCl until the absorbance at 280 nm reached baseline values. Finally, SFL activity was eluted with a 50 ml linear gradient from 0.4 M to 1.5 M NaCl, in the same buffer, at 1 ml/min.

In parallel HGF/SF purification from 3-day serum-free MRC5 conditioned medium (800 ml) was also carried out by heparin-Sepharose, Bio Rex 70, Mono Q chromatographies and RP-HPLC. Purified HGF/SF, from RP-HPLC fractions, was detected by ELISA using specific polyclonal antibodies against human HGF/SF. hrHGF/SF.

**ELISA for HGF/SF detection**

A direct ELISA for HGF/SF was carried out as follows: microtiter plates were coated overnight at 4°C with the RP-HPLC fractions that had been previously freeze-dried and resuspended in 100 ml 0.1 M sodium carbonate buffer, pH 9.6. The plates were washed three times in 0.02 M PBS, pH 7.4 (containing 0.1% Tween-20 and 0.02% sodium azide); they were then incubated for 90 minutes at 37°C with polyclonal rabbit antibodies against human HGF/SF, diluted 1/500 in PBS buffer containing 1% normal goat serum.

After aspiration and three washes, the plates were incubated for another 90 minutes at 37°C with a goat IgG anti-rabbit IgG conjugated to alkaline phosphatase (Sigma), diluted to 1/1000. Finally, the plates were aspirated, washed and the enzymatic activity was assessed by the addition of the substrate (1 mg/ml p-nitrophenylphosphate disodium in 0.05 M carbonate buffer, pH 9.8).

The reaction was followed by reading the absorbance every 30 minutes at 410 nm using a Dynatech ELISA reader. HGF/SF content of the different fractions was estimated by comparison with control hrHGF/SF.

**Northern blot experiments**

E-NBT-II cells were analyzed by northern blotting to detect the presence of endogenous HGF/SF transcripts. MRC5 cells and MDCK cells were used, respectively, as positive and negative controls.

mRNAs were prepared using the FastTrack kit (Invitrogen Co, British Biotechnology Products Ltd). A 1 µg sample of mRNA was electrophoresed in formaldehyde-1.2% agarose gel and blotted onto Hybond-N membrane filters (Amersham, France, Les Ullis). Filters were hybridized with cDNA probes under stringent conditions for 16 hours at 42°C in 50% formamide (Fluka, Buchs, Switzerland), 2x SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7), 5x Denhardt, 0.1% SDS and 100 µg/ml sonicated salmon sperm DNA.

**Probes and oligonucleotides**

The rat HGF/SF probe is the complete 2.3 kb coding sequence and the rat met probe is a NotI-XhoI, 2 kb CDNA insert, which includes the region coding for the extracellular domain of the receptor. The HGF-like probe is the rat homologue of the mouse HGF-like domain coding for kringle 3 and 4 of the putative growth factor (Degen et al., 1991) and was a kind gift from Dr S. Degen (University of Cincinnati, Cincinnati, Ohio). The size of the detected message was estimated by comparison with a 0.24 to 9.5 kb RNA ladder (Gibco BRL). Plasmid containing the rat cDNA coding sequence for glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a probe for the internal control for the mRNA preparation (Fort et al., 1985).

Oligonucleotide primers corresponding to sequences entirely conserved between human and rat HGF/SF were used for reverse transcriptase-PCR (RT-PCR) experiments according to a published protocol (Radvanyi et al., 1993). Sense oligonucleotide (563 to 583) and antisense oligonucleotide (622 to 641) were synthesized according to the rat HGF/SF cDNA sequence (Tashiro et al., 1990).

**RESULTS**

**In vitro properties of the selected metastatic M-NBT-II variant**

The metastatic variant M-NBT-II selected after two passages in vivo, is of mesenchymal morphology and exhibits enhanced tumorigenic behavior when compared with parental E-NBT-II cells. The time of appearance of a 200 mm³ tumor is reduced from 42 days to 10 days (Fig. 1A). Lymph node micrometastases were obtained earlier for M-NBT-II cells as a consequence of the reduction of the latency for tumor growth. In all cases, micrometastases were detected when the tumor reached approximately 200 mm³. Subsequent passages of M-NBT-II cells (M-NBT-II bis) in nude mice did not further increase the tumorigenicity, indicating that the selected variant exhibits stable in vivo behavior (Fig. 1A).

**In vitro behavior and invasive properties of M-NBT-II cells**

The in vitro growth properties of E-NBT-II, E/M-NBT-II and M-NBT-II cells were similar, and no significant differences in growth rates were observed in FCS-containing medium (doubling time 24 hours) (Fig. 1B).

Fig. 2A and B shows that M-NBT-II cells are scattered as compared to E-NBT-II cells from which they originate. Immuno-staining experiments with antibodies directed against desmosomal components showed that 90% of M-NBT-II cells had lost their desmosomal cell-cell contacts as compared to 1-5% for E-NBT-II cells (data not shown).

Growth of M-NBT-II cells in 3-D collagen gels result in a three-dimensional dissociated structure, whereas E-NBT-II cells grow essentially as compact spheroids (Fig. 2C and D); furthermore, unlike E-NBT-II parental cells, M-NBT-II cells secreted a considerable amount of 92 kDa and 62 kDa gelatinase activities as revealed by zymography (Belluscio, S. and Jouanneau, J., unpublished results; Jouanneau et al., 1991). These results are consistent with the invasive and metastatic phenotype displayed by M-NBT-II cells in vivo.

**Metastatic M-NBT-II cells produce and secrete a scatter activity (SFL)**

Among the growth factors tested that dissociate the E-NBT-II cells, HGF/SF is the only one that also dissociates the MDCK cells; thus we have comparatively tested the biological activity of HGF/SF and of M-NBT-II conditioned medium in all the experiments. M-NBT-II cells produce a dissociating activity that induces, in vitro, the scattering of clustered E-NBT-II cells (Fig. 3C and D). Experiments carried out with HGF/SF containing medium or recombinant HGF/SF on NBT-II cells give similar results, as previously reported (Belluscio et al., 1994). Notably, the medium conditioned by M-NBT-II cells induces the scattering of epithelial MDCK cells, which are known to
Fig. 1. In vivo selection of the metastatic variant M-NBT-II from NBT-II carcinoma cells. Left: M-NBT-II metastatic variant was selected after an in vivo passage of a first heterogeneous metastasis (E/M-NBT-II) obtained in a nude mouse. Right: growth properties. (A) Tumor progression in nude mouse of E-NBT-II, E/M-NBT-II, M-NBT-II and M-NBT-II bis; (B) in vitro growth of E-NBT-II, E/M-NBT-II and M-NBT-II tumor-derived cells in standard medium (e+6, 10⁶).

Fig. 2. Morphology of M-NBT-II versus E-NBT-II. (A) E-NBT-II cells and (B) M-NBT-II cells culture in standard medium. (C) E-NBT-II cells and (D) M-NBT-II cells culture in 3-D collagen gel. Bar, 100 μm.
Scatter factor-like factor and tumor cells

The M-NBT-II supernatant was also able to dissociate NBT-II spheroids grown in collagen (data not shown). Conversely, the medium conditioned by E-NBT-II cells was not active in similar experiments (data not shown). The scattering activity produced by M-NBT-II cells being reminiscent of the HGF/SF activity, we have designated this activity, scatter factor-like (SFL).

Biochemical characteristics of SFL

Using the MDCK cell dissociation assay, the biological activity of the M-NBT-II supernatant is at least 3 times less active than the MRC5 supernatant. This result may be explained by the fact that MRC5 supernatant is a 3-day serum-free conditioned medium, whereas M-NBT-II conditioned medium is collected after 24 hours. It was not possible to increase the biological activity of M-NBT-II supernatant by incubating M-NBT-II cells with serum-free medium for more than one day. This was due to the fact that M-NBT-II cells detach from plastic and that the 48 hour serum-free conditioned medium exhibited even less activity than the 24 hour conditioned medium.

Attempts to concentrate the M-NBT-II supernatant by ultracentrifugation on a 10 kDa membrane (Ultrasart System, Sartorius) resulted in a decrease in activity by more than 50% suggesting non-specific binding to the membrane, the activity was detected only in the concentrated fraction and the use of Triton X-100 did not improve the yield of SFL activity. Concentration experiments of small volume (2 ml) were also carried out using a Centricon microconcentrator (Amicon) with several cut-off membranes (3, 10, 30 and 100 kDa). The activity was only found in the concentrate with the 3, 10 and 30 kDa membranes but was also present in the dialysate with the 100 kDa cut-off membrane, suggesting that the apparent molecular mass of SFL factor is around 100 kDa. This result is in agreement with the molecular mass estimated from the elution volume of the SFL activity after a Sephacryl 200 gel filtration column (Pharmacia) carried out with the Bio Rex 70 partially purified fraction. Interestingly, SFL factor and HGF/SF behave similarly when used in the same concentration experiments and gel filtration chromatographies (data not shown).

Previous reports have demonstrated that HGF/SF can be purified either on heparin-Sepharose, by exploiting its high affinity for heparin (Weidner et al., 1990; Rubin et al., 1991), or by ionic interactions with both cation (Gherardi et al., 1989; Weidner et al., 1990; Rubin et al., 1991;) and anion (Gherardi et al., 1989) resins. HGF/SF has also been purified by reverse-phase chromatography (Gherardi et al., 1989; Rubin et al., 1991). We carried out, in parallel, affinity chromatographies on heparin-Sepharose, with both M-NBT-II and MRC5 (human HGF/SF-containing medium) conditioned media. Table 1 shows that SFL factor binds to heparin and can be eluted with the same salt concentration as HGF/SF (0.9-1.3 M NaCl). However, yields and specific activities were not comparable. The low yield obtained for SFL activity (14% instead

---

Fig. 3. SFL factor dissociates E-NBT-II and MDCK cells. MDCK cells culture in standard medium (A) or in M-NBT-II conditioned medium (B). E-NBT-II cells cultured in standard medium (C) or in M-NBT-II conditioned medium (D). Bar, 100 µm.
of 80% for HGF/SF) could not be improved by the addition of 0.1% Triton X-100 in both the culture media and buffers. This discrepancy could be due to the different biochemical behavior of SFL factor and HGF/SF. Furthermore, a greater number of contaminating proteins were found in the SFL active fractions than in HGF/SF active fractions, according to the 3.39 units/mg specific activity calculated for SFL and 489 units/mg for HGF/SF (Table 1).

In order to improve the recovery of SFL activity, we carried out another purification strategy based on Bio Rex 70 cation chromatography, followed by either Mono Q anion chromatography or C18 reverse-phase chromatography (Fig. 4A, B...
The SFL was eluted from the Bio Rex column between 0.4 M and 0.6 M NaCl with an 8.9% yield (specific activity of 4.3 units/mg) (Fig. 4A and Table 2). At this step the use of Triton X-100 did not give a better yield. The SFL activity present in the active Bio Rex fractions was totally abolished after a 5-minute incubation at 100°C. The active fractions, after recovery from the Bio Rex, were loaded on the Mono Q column. SFL factor was eluted between 0.25 M and 0.37 M NaCl with a 47% yield (2% of the initial activity, purification factor of 49.7) (Fig. 4B and Table 2). A better purification was obtained on a C18 reverse-phase column, as was observed after SDS-PAGE and SFL activity was eluted with 37% acetonitrile (Fig. 4D and C, and Table 3). At this stage, the yield was very low (0.05% of the initial activity) as a result of protein denaturation after lyophilization. The different steps of SFL factor purification are compiled in Table 2.

Comparative reverse-phase chromatography with both active HGF/SF heparin fractions from MRC5 conditioned medium (13,000 units) and recombinant HGF/SF (100 ng) resulted in similar findings: the HGF/SF activity could not be detected after lyophilization. However, specific antibodies against HGF/SF, used in an ELISA test, show that HGF/SF protein was eluted with 40% acetonitrile.

SDS-PAGE, in reducing conditions, of heparin-Sepharose (data not shown) or Bio Rex 70 active fractions showed numerous bands. However, after Mono Q chromatography or RP-HPLC, it was obvious that a 90-100 kDa band was specifically found in the active fractions (Fig. 4-D). It was not possible to recover SFL activity from a native gel by electroelution, as probably not enough activity was available.

Table 3 summarizes the comparative results obtained for SFL factor and HGF/SF-containing medium. These two factors have very similar chromatographic behavior.

### SFL factor and HGF/SF exhibit different properties

A specific experiment to test HGF/SF activity is induction of tubulogenesis (Fig. 5C), which cannot be induced by several other growth factors as reported earlier (Montesano et al., 1991a). We have been able to show unambiguously that M-NBT-II conditioned medium or SFL-purified fractions cannot induce tubulogenesis (Fig. 4B) in MDCK cells grown on 3-D collagen gels.

It has been reported that epithelial tubular morphogenesis requires collagenase activity as, in this system, exogenously

---

**Table 2. SFL-factor purification**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein content (μg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/μg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-NBT-II conditioned medium</td>
<td>73,700</td>
<td>24,600</td>
<td>0.33</td>
<td>100.00</td>
<td>1.0</td>
</tr>
<tr>
<td>0.4-0.6 M NaCl (18 ml)</td>
<td>504</td>
<td>2189</td>
<td>4.34</td>
<td>8.90</td>
<td>13.2</td>
</tr>
<tr>
<td>Mono Q 0.25-0.37 M NaCl (4 ml)</td>
<td>30</td>
<td>492</td>
<td>16.40</td>
<td>2.00</td>
<td>49.7</td>
</tr>
<tr>
<td>RP-HPLC 37% acetonitrile (200 μl)</td>
<td>2.6</td>
<td>12</td>
<td>4.60</td>
<td>0.05</td>
<td>14.0</td>
</tr>
</tbody>
</table>

---

Fig. 5. SFL does not induce tubulogenesis. MDCK spheroids grown within collagen gel in standard medium (A), in the presence of M-NBT-II conditioned medium (B) or in the presence of recombinant HGF/SF (C) (same tubulogenic induction is observed with HGF/SF containing MRC5 or NIH 3T3 conditioned media). Bar, 135 μm.
added synthetic collagenase inhibitor completely prevented the formation of branching tubules, resulting instead in the development of cyst-like structures (Montesano et al., 1991a). To test this possibility, we looked for the presence of collagenase inhibitors in the M-NBT-II supernatant. A mixture of MRC5 HGF/SF-containing medium and M-NBT-II supernatant (1:1, v/v) was thus used; no tubulogenesis inhibition was observed, indicating that the absence of biological activity of the SFL factor in this system is not due to collagenase inhibitors secreted by M-NBT-II cells (data not shown). Thus, biologically active SFL is not tubulogenic per se.

Polyclonal-blocking antibodies raised against rat HGF/SF were not able to inhibit the dissociating activity contained in both M-NBT-II conditioned medium (not shown) and partially purified fractions (Fig. 6A and B). Different antibody concentrations have been used (1-5 μg/ml); 1 μg/ml of blocking antibodies was sufficient to inhibit completely the scatter effect of rat recombinant HGF/SF (data not shown) and of NIH 3T3 ras D4 conditioned medium (Fig. 6C and D). This culture supernatant contains a similar amount of scatter activity to that produced by M-NBT-II cells.

Polyclonal-blocking antibodies raised against rat HGF/SF were not able to inhibit the dissociating activity contained in both M-NBT-II conditioned medium (not shown) and partially purified fractions (Fig. 6A and B). Different antibody concentrations have been used (1-5 μg/ml); 1 μg/ml of blocking antibodies was sufficient to inhibit completely the scatter effect of rat recombinant HGF/SF (data not shown) and of NIH 3T3 ras D4 conditioned medium (Fig. 6C and D). This culture supernatant contains a similar amount of scatter activity to that produced by M-NBT-II cells.

Table 3. SFL-factor and HGF/SF comparative purification steps

<table>
<thead>
<tr>
<th>Step</th>
<th>SFL-factor</th>
<th>HGF/SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin-Sepharose*</td>
<td>0.65-1.30 M†‡</td>
<td>0.65-1.20 M†‡</td>
</tr>
<tr>
<td>Bio Rex 70*</td>
<td>0.40-0.60 M†‡</td>
<td>0.45-0.70 M†‡</td>
</tr>
<tr>
<td>Mono Q*</td>
<td>0.25-0.40 M†‡</td>
<td>0.30-0.40 M†‡</td>
</tr>
<tr>
<td>RP-HPLC†‡</td>
<td>37%*‡</td>
<td>40%§‡</td>
</tr>
</tbody>
</table>

*Recovery of the growth factor is determined on the basis of the biological dissociating activity.
†NaCl concentration.
‡Most of the activity (>90%) is eluted between 0.9 and 1.2 M NaCl.
§Acetonitrile % for HGF/SF recovery is based on the ELISA test (see Material and Methods).

Finally, northern blot experiments, using the complete HGF/SF rat cDNA probe at high and low stringencies, were unsuccessful in detecting HGF/SF transcripts from M-NBT-II mRNAs (Fig. 7). Three bands corresponding to the transcripts already reported were detected in MRC5 mRNAs (Hartmann et al., 1992). In addition, NBT-II and M-NBT-II cells expressed c-met, the HGF/SF receptor (Bottaro et al., 1991; Naldini et al., 1991a,b). HGF-like transcripts were not detected in M-NBT-II cells (data not shown). Furthermore, RT-PCR experiments for HGF/SF did not result in the detection of specific sequences in both E-NBT-II and M-NBT-II cells, whereas a strong amplification was obtained for MRC5 cells (not shown).
The effect of HGF/SF on MDCK cell dissociation has been reported to increase tumorigenic behavior in vivo. We developed an in vivo approach allowing the selection of metastatic cells that spontaneously acquire, in vitro, a fibroblastic-like phenotype in vitro (Jouanneau et al., 1994). These experimental results, obtained with the NBT-II cell system, determined that we should investigate whether an endogenous growth/scatter factor, spontaneously produced by a subset of cells during tumor progression, could mimic the effect of the transfected aFGF on NBT-II cells.

We developed an in vivo approach allowing the selection of metastatic cells that spontaneously acquire, in vitro, a fibroblastic morphology and produce such a growth/dissociating factor. Most of the metastatic cells obtained in this way had an epithelial or a heterogeneous morphology, indicating that the expression of the putative growth/dissociating factor might be transient. However, we were able to select, in vivo, a stable metastatic NBT-II variant (M-NBT-II) that secretes a dissociating activity, termed SFL activity, reminiscent of that of HGF/SF. The M-NBT-II cells acquired increased tumorigenic and metastatic properties as compared to the parental cells.

SFL factor shares some biological properties with HGF/SF such as induction of MDCK and NBT-II cell scattering in vitro. The effect of HGF/SF on MDCK cell dissociation has been reported to be specific; other factors such as insulin, transferrin, platelet-derived growth factor, macrophage colony-stimulating factor, basic and acidic fibroblast growth factors and also epidermal growth factor were not able to induce the scattering of MDCK cells (Rosen et al., 1990).

HGF/SF is mainly a paracrine factor produced by cells of mesenchymal origin, which acts on epithelial cells (Stoker et al., 1987; Weidner et al., 1990; Sonnenberg et al., 1993); however, one case of autocrine activity has already been described (Adams et al., 1991). HGF/SF has been reported to be a morphogenetic (Montesano et al., 1991) and angiogenic factor in vitro (Bussolino et al., 1992), potentially involved in early development (Stern et al., 1990; Defrances et al., 1992; Sonnenberg et al., 1993).

HGF/SF is produced in an immature form and is extracellularly processed by proteolytic cleavage to form a glycosylated disulfide-linked heterodimer, consisting of a 62 kDa heavy chain and a 34/32 kDa light chain (Matsumoto and Nakamura, 1992; Weidner et al., 1990). Its cellular receptor has been identified as the c-met proto-oncogene product (Bottaro et al., 1991; Naldini et al., 1991a,b). The mature c-met protein is a heterodimer of 190 kDa composed of 145 kDa β and 50 kDa α subunits. The β-chain extends over the membrane and contains the tyrosine kinase catalytic domain, whereas the α-chain remains extracellular (Park et al., 1987; Giordano et al., 1989).

HGF/SF and its c-met receptor could be critical in pathological processes such as tumor progression; HGF/SF dissociates several carcinoma cell lines in vitro (Jiang et al., 1993; Weidner et al., 1990) and cells expressing both HGF/SF and c-met protein are tumorigenic in nude mice (Rong et al., 1993). This suggests that an autocrine transformation mechanism could be relevant in such processes. It has been reported recently that three lung carcinoma cell lines expressed autocrine HGF/SF (Tsao et al., 1993). Significant amounts of human HGF/SF have been found in the pleural of patients where malignant invasion had occurred (Kenworthy et al., 1992) and the c-met protein is increased more than 100-fold in human thyroid carcinoma cells versus their normal counterparts (Di Renzo et al., 1991). However, HGF/SF has also been shown to inhibit growth in some cancer cell lines both in vitro (Higashio et al., 1990; Shiota et al., 1992; Jiang et al., 1993) and in vivo (Shiota et al., 1992).

It has been reported recently, that HGF/SF decreases the expression of tissue inhibitors of metalloproteinases in human renal cancer cells (Nakayama et al., 1993). Interestingly, M-NBT-II cells expressed high amounts of metalloproteinases; this expression could be stimulated by the SFL factor, leading to increased tumorigenic behavior in vivo.

DISCUSSION

Previous experiments carried out in our laboratory have shown that NBT-II cells, made autocrine for aFGF, have a fibroblastic-like phenotype in vitro (Jouanneau et al., 1991), and have acquired new invasive (Tucker et al., 1991) and tumorigenic properties (Jouanneau et al., 1994). These experimental results, obtained with the NBT-II cell system, determined that we should investigate whether an endogenous growth/scatter factor, spontaneously produced by a subset of cells during tumor progression, could mimic the effect of the transfected aFGF on NBT-II cells.

We developed an in vivo approach allowing the selection of metastatic cells that spontaneously acquire, in vitro, a fibroblastic-morphology and produce such a growth/dissociating factor. Most of the metastatic cells obtained in this way had an epithelial or a heterogeneous morphology, indicating that the expression of the putative growth/dissociating factor might be transient. However, we were able to select, in vivo, a stable metastatic NBT-II variant (M-NBT-II) that secretes a dissociating activity, termed SFL activity, reminiscent of that of HGF/SF. The M-NBT-II cells acquired increased tumorigenic and metastatic properties as compared to the parental cells.

SFL factor shares some biological properties with HGF/SF such as induction of MDCK and NBT-II cell scattering in vitro. The effect of HGF/SF on MDCK cell dissociation has been reported to be specific; other factors such as insulin, transferrin, platelet-derived growth factor, macrophage colony-stimulating factor, basic and acidic fibroblast growth factors and also epidermal growth factor were not able to induce the scattering of MDCK cells (Rosen et al., 1990).
(Hartmann et al., 1992). These results also allow us to exclude the possibility that SFL factor is a HGF/SF variant resulting from a point mutation, a deletion, or an alternative splicing. SFL factor is a new factor sharing some but not all the biological activities of HGF/SF.

Recent reports have described the cloning of a putative HGF-like factor (Degen et al., 1991) and of a met-like receptor (Ronsin et al., 1993), based on their DNA sequence homologies with HGF/SF and met, respectively. Nevertheless, so far, the biological activity of this putative factor and this receptor are not known. On the basis of northern blotting, we have excluded the possibility that SFL factor may be the HGF-like factor (data not shown).

Although HGF/SF and SFL are not identical, from their scattering activity and apparent size we propose that the SFL factor may be a new member of the HGF/SF family that could be involved in tumor progression. On this assumption, SFL could be expressed transiently during the course of carcinoma progression and, as such, may represent a prognostic factor.

We thank M. F. Poupon and Y. Bourgeois for the animal facility and the nude mice injections; K. M. Weidner, W. Birchmeier and R. Montesano, D. Soyez for their help and advice during the initial steps of this work; T. Nakamura for the recombinant HGF/SF and the antibodies; G. Gaudino and P. Comoglio for the HGF/SF and c-met probes; S. Degen for the HGF-like probe; W. Franke for the antibodies against desmoplakins; A. M. Vallés, B. Barbour and P. Wyplosz for critical reading of the manuscript; and D. Morineau for the photographs. This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS), the Association pour la Recherche sur le Cancer (ARC-6465), the Ligue Nationale Française contre le Cancer (National Committee and Committee of Paris), the Groupement des Entreprises Françaises contre le Cancer (GEFLUC), the National Cancer Institute (NIH, 2R01 CA 9417-04) and the Human Frontier Science Program Organization.

REFERENCES


(Received 9 December 1993 - Accepted 26 January 1994)