

# Expression of a Human Hepatocyte Growth Factor/Scatter Factor cDNA in MDCK Epithelial Cells Influences Cell Morphology, Motility, and Anchorage-independent Growth

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**Abstract.** The addition of exogenous hepatocyte growth factor (HGF)/scatter factor (SF) to MDCK epithelial cells results in fibroblastic morphology and cell motility. We generated HGF/SF producing MDCK cells by transfection with an expression plasmid containing human HGF/SF cDNA. Production of HGF/SF by these cells induced a change from an epithelial to a fibroblastic morphology and increased cell motility. In ad-

dition, the HGF/SF producing cells acquired efficient anchorage-independent growth in soft agar but did not form tumors in nude mice. The morphological change and the stimulation of the anchorage-independent growth were prevented by anti-HGF/SF antibody, suggesting that the factor is secreted and then exerts its effects through cell surface receptors.

CELL dispersion and motility are required for normal embryogenesis and tissue remodeling, and are also important steps in the invasion of tumor cells (Rosen and Goldberg, 1989). The molecular mechanisms responsible for the induction of cell dispersion and cell motility remain unclear. Several factors affecting cell motility have been discovered (Rosen and Goldberg, 1989). Among them, scatter factor (SF),<sup>1</sup> a mesenchymal cell-derived protein, dissociates epithelial cell colonies to individual cells and stimulates the migration of epithelial cells (Stoker and Perryman, 1985; Stoker et al., 1987; Gherardi et al., 1989). Since implantation of SF-producing human fibroblasts into chick embryos caused abnormal development, it was suggested that SF is involved in embryogenesis (Stern et al., 1990). Purified SF promotes the invasiveness into collagen matrices of a number of human carcinoma cell lines, suggesting that SF is involved in the invasion of tumor cells (Weidner et al., 1990).

Recently, scatter factor has been found to be identical to hepatocyte growth factor (HGF) (Weidner et al., 1991). HGF was originally identified as a potent mitogen for hepatocytes in primary culture (Gohda et al., 1988; Nakamura et al., 1987; Zarnegar and Michalopoulos, 1989). HGF is also mitogenic in melanocytes and endothelial and epithelial cells (Rubin et al., 1991). Moreover, the same factor was purified from the culture medium of human embryonic lung fibroblasts as a cytotoxic factor in some tumor cell lines (Higashio et al., 1990). Thus, HGF has a broad spectrum

of activities among its various target cells. To simplify the multiple names of this factor, we will refer to it as HGF/SF in this paper. We and others have cloned and sequenced the cDNA for human and rat HGF/SF (Miyazawa et al., 1989; Nakamura et al., 1989; Tashiro et al., 1990; Okajima et al., 1990). Human HGF/SF is synthesized as a single 90-kD precursor, whose NH<sub>2</sub>-terminal 31 amino acid residues probably function as a signal peptide (Yoshiyama et al., 1991). The precursor is glycosylated and cleaved at a specific proteolytic site to yield a 65-kD heavy chain and a 35-kD light chain, which are linked together by a disulfide bond.

The dog kidney epithelial cell line, MDCK, is a highly sensitive target and has been used in a bioassay for the factor. In tissue culture, MDCK cells form tight epithelial monolayers with junctional complexes (McRoberts et al., 1981). When added to cultured MDCK cells, purified SF causes the breakdown of intercellular junctions and a transition from an epithelial to a fibroblastic morphology (Gherardi et al., 1989; Weidner et al., 1990). It also stimulates the motility of MDCK cells (Stoker, 1989).

To determine the potential autocrine role of HGF/SF in the acquisition of scattering and motile phenotypes at the single cell level, we introduced a plasmid that directs the constitutive synthesis of human HGF/SF into MDCK epithelial cells. Cells producing human HGF/SF were converted morphologically to fibroblast-like scattered cells and became motile. These cells also acquired efficient growth property in soft agar. Since the morphological change and the stimulation of the growth in soft agar were prevented in the presence of anti-HGF/SF antibody, the factor is secreted and then exerts its effects through receptors on the cell surface.

1. *Abbreviations used in this paper:* HGF, hepatocyte growth factor; SF, scatter factor.

## Materials and Methods

### Materials

Reagents were obtained as follows: restriction endonucleases from Takara Shuzo Co. (Kyoto, Japan) and Toyobo Co. (Osaka, Japan); the Klenow fragment of *Escherichia coli* DNA polymerase I from Boehringer Mannheim (Indianapolis, IN); Geneticin (G418) from Gibco Laboratories (Grand Island, NY); heparin-Sepharose from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ); human EGF from Wakunaga Co. (Osaka, Japan). Recombinant human HGF/SF and anti-human HGF/SF polyclonal and monoclonal antibodies were provided from the Research Center, Mitsubishi Kasei Co. (Yokohama, Japan) (Strain et al., 1991). An ELISA kit for human HGF/SF was provided by Dr. S. Shin, Otsuka Assay Laboratories, Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan) (Tsubouchi et al., 1991).

### Plasmid Construction

The N2 vector (Armentano et al., 1987), containing the neomycin-resistance (*Neo<sup>R</sup>*) gene, was provided by Dr. E. Gilboa (Memorial Sloan-Kettering Cancer Center). The 2.8-kbp BamHI fragment, containing the entire coding region of human HGF/SF cDNA, was excised from the human HGF expression plasmid pSR $\alpha$ -hHGF (Miyazawa et al., 1991), and ligated into the BamHI site of pUC18. In the plasmid, the SalI site in the polylinker sequence of the vector was located upstream of the 5' end of the cDNA. The pUC18-hHGF plasmid was partially digested at the one BamHI site corresponding to the 3' end of the cDNA. Two nucleotides were added to the cleaved BamHI site using the Klenow fragment of DNA polymerase I. The linear DNA was digested with SalI and the fragment containing the cDNA was isolated. The 0.6-kbp HindIII-XhoI fragment, containing the SR $\alpha$  promoter sequence, was excised from the pcDL-SR $\alpha$ 296 vector (Takebe et al., 1988), and ligated into the HindIII-XhoI site of pBluescript. The plasmid was digested with BamHI and two nucleotides were inserted into the site using the Klenow fragment of DNA polymerase I. The linear DNA was digested with XhoI and the fragment containing the SR $\alpha$  promoter sequence was isolated. The resultant two fragments were ligated into the XhoI site of the N2 vector which was filled in with two nucleotides.

### Cell Culture and Transfection

MDCK cells were obtained from American Type Culture Collection (Rockville, MD). A subclone which exhibited epithelial morphology was selected by limiting dilution and transfected. The cell line was cultured in DME supplemented with 5% FBS. Cells were transfected by electroporation. 15  $\mu$ g of plasmid DNA was mixed with  $5 \times 10^6$  cells in 0.5 ml DME and pulsed once at 1,000 V (capacitance, 35  $\mu$ F) in a cuvette (0.4 cm gap) at room temperature. 3 d after transfection, cells were cultured in selective medium containing the neomycin analogue G418 (800  $\mu$ g/ml). After 14–18 d, resistant colonies were selected and cultured in selective medium.

### ELISA and Immunoblot Analysis of Human HGF/SF

The level of HGF/SF in the conditioned medium from each transfectant was quantitatively measured using an ELISA kit with native human HGF as a standard. The cells were grown to confluence in 24-well plates and the secreted HGF/SF in 1 ml of the medium after 24 h culture was measured by ELISA. For immunoblot analysis, HGF/SF was partially purified by heparin-Sepharose. Conditioned medium (2 ml) was incubated with 10  $\mu$ l of heparin-Sepharose (1:1, swollen in 20 mM Tris-Cl (pH 7.4) containing 0.15 M NaCl) overnight at 4°C with gentle agitation. The heparin-Sepharose was collected by centrifugation, and washed with 20 mM Tris-Cl (pH 7.4) containing 0.15 M NaCl and 0.013% CHAPS. Sample buffer (20 mM Tris-Cl (pH 6.8) containing 2% SDS, 2% 2-mercaptoethanol, 40% glycerol and 0.2% bromophenol blue) was added to the washed heparin-Sepharose, and boiled for 5 min. The eluted proteins were separated by SDS-PAGE on a 10% polyacrylamide gel. After SDS-PAGE, proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Cambridge, MA). The filter was immunoblotted as described previously (Miyazawa et al., 1991). The color was developed by soaking the filter in TMB (Transgenic Science Inc., Worcester, MA).

### Soft Agar Colony Assay

Cells,  $2 \times 10^3$ , were suspended in 1 ml DME containing 10% FBS and 0.33% Noble agar (Difco Laboratories, Detroit, MI), and plated onto the same medium containing 0.5% Noble agar to each well of a 6-well plate.

Human HGF/SF, human EGF, or polyclonal anti-HGF/SF antibody was added to the cell suspension before they were plated. 1 d after incubation, 1 ml DME containing 10% FBS was added, and the medium was changed at intervals of 7 d. The number of colonies greater than 0.2 mm in diameter was counted at 28 d.

### Cell Motility Assay

The assay was performed by standard methods using blind-well chambers (B-02; Labo Science, Tokyo, Japan) and 8  $\mu$ m nucleopore filters (Stoker, 1989). Filters soaked in collagen type IV were placed between the lower wells containing 0.2 ml of DME with 10% FBS and the upper wells containing 0.1 ml of  $10^6$  cells/ml. After incubation for 5 h, the filters were fixed and stained with hematoxylin, and nonmigrating cells that had accumulated on the upper surface were removed. Nucleated cells that migrated through the filters were counted on micrographs at a magnification of  $\times 100$ .

## Results

### Stimulation of Anchorage-independent Growth of MDCK Cells by HGF/SF

HGF/SF was characterized by its ability to induce the disruption and scattering of epithelial colonies and to increase the movement of individual MDCK cells (Stoker and Perryman, 1985). These characteristic changes occur without affecting growth of the cells (Gherardi et al., 1989), even though HGF/SF is mitogenic in some other epithelial lines (Rubin et al., 1991). Although it has been reported that MDCK cells form colonies in semisolid medium (Stiles et al., 1976), the effect of HGF/SF on anchorage-independent growth has not been extensively studied. We therefore investigated whether HGF/SF stimulates the growth of MDCK cells in soft agar. MDCK cells formed colonies in soft agar with an efficiency of  $\sim 5\%$ , even in the absence of HGF/SF. However, the growth of MDCK cells in soft agar was slow and colonies were very small even after 28 d in culture (smaller than 50  $\mu$ m in diameter). In the presence of HGF/SF the growth of MDCK cells in soft agar was stimulated in a dose-dependent manner between 2 and 200 ng/ml (Table I). Colonies were much larger after 28 d in culture than those of untreated MDCK cells.

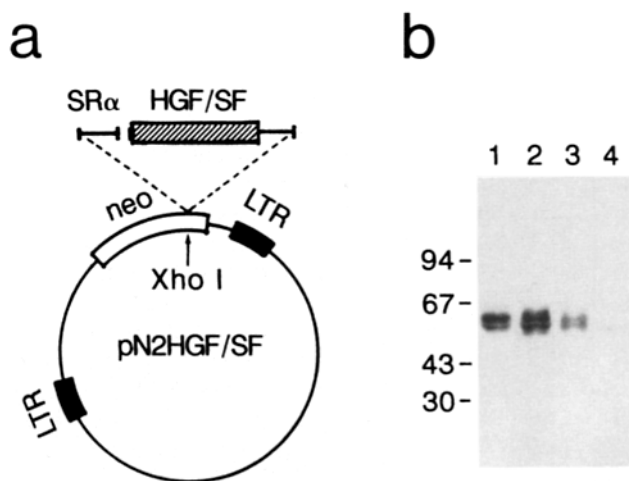
### Production of HGF/SF by MDCK Cells Transfected with Human HGF/SF cDNA

An expression plasmid was constructed in which the human HGF/SF cDNA sequence was placed downstream of the

**Table I. Stimulation of Growth of MDCK Cells in Soft Agar in Response to Exogenously Added HGF/SF**

Concentration of HGF/SF added	Number of Colonies (Efficiency)
ng/ml	%
0	0 (0)
2	0 (0)
5	4 (0.2)
20	20 (1.0)
50	24 (1.2)
100	33 (1.6)
200	38 (1.9)
400	37 (1.8)

Colonies larger than 0.2 mm in diameter were counted after 28 d in culture. Note that MDCK colonies were detected in soft agar even in the absence of HGF/SF but they were very small (smaller than 50  $\mu$ m). Numbers are averages of triplicates.



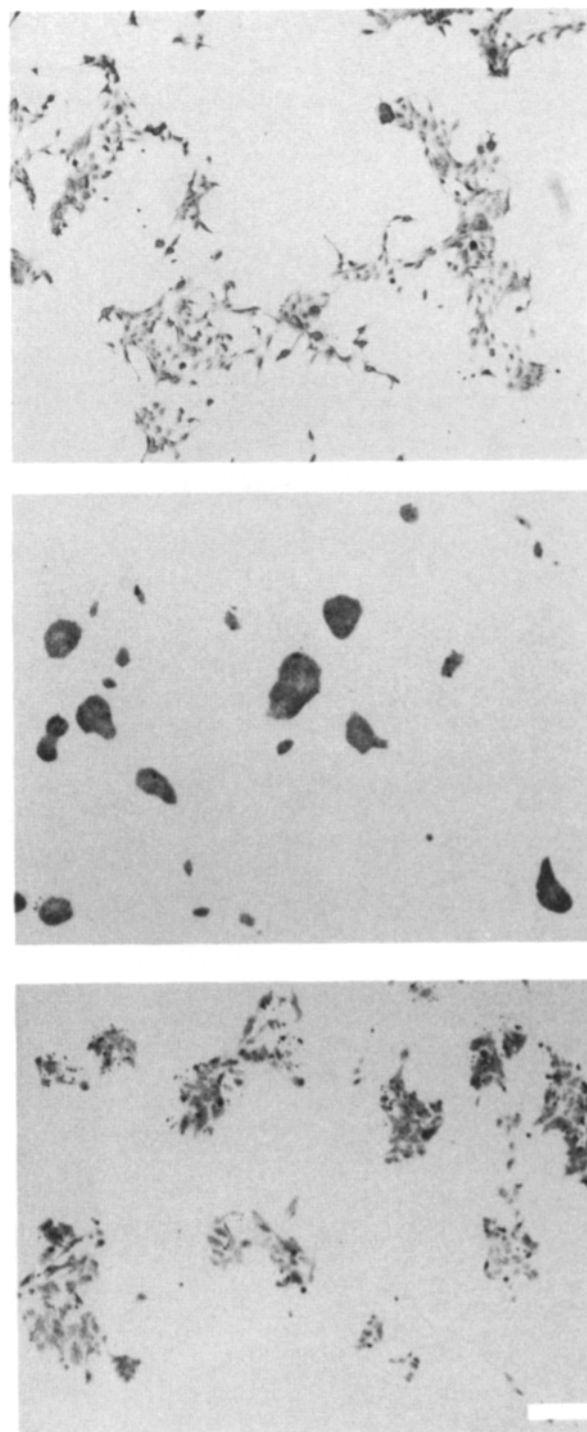
**Figure 1.** Expression of human HGF/SF. (a) Schematic diagram of the pN2HGF/SF expression vector. The dashed box represents the coding region for human HGF/SF. (b) Immunoblot analysis. Conditioned media of HGF/SF producing clones A4 (lane 2), A1 (lane 3), and parental cells (lane 4) were analyzed. Recombinant human HGF/SF was blotted as a control (lane 1). The molecular mass standards were phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), and carbonic anhydrase (30 kD).

SR $\alpha$  promoter (Fig. 1 a). This plasmid contains the neomycin phosphotransferase gene which confers G418 resistance. The plasmid was introduced into the MDCK cells by electroporation, and clones resistant to 800  $\mu$ g/ml of G418 were selected and expanded. The ability of the clones to secrete human HGF/SF into the culture medium was assayed by an ELISA which was sensitive enough to detect 0.3 ng/ml of HGF/SF. 6 out of 17 clones secreted human HGF/SF at a concentration over 0.3 ng/ml per 24 h. To confirm that the molecules detected by ELISA were intact HGF/SF, the conditioned media from two clones were concentrated by heparin-Sepharose and assayed by immunoblotting. The mAb used in this assay recognizes a peptide sequence in the heavy chain region of HGF/SF (Miyazawa et al., 1991). As shown in Fig. 1 b, a band with a molecular mass of  $\sim$ 65 kD was detected in each clone. The molecular size corresponded to that of the heavy chain of recombinant human HGF/SF (Strain et al., 1991). The results indicated that the expression plasmid containing the sequence of the cDNA faithfully generated the intact form of HGF/SF.

#### **Morphology and Motility of HGF/SF Producing MDCK Cells**

All six clones that secreted significant quantities of HGF/SF exhibited fibroblast-like scattered morphology. The morphology of one of the HGF/SF producing clones is shown in Fig. 2. Another five clones exhibited the same fibroblast-like morphology (not shown). This morphology was similar to that of MDCK cells treated exogenously by HGF/SF, but was readily distinguishable from the parental MDCK cells. Four clones secreting varying levels of HGF/SF were further studied (Table II).

It was demonstrated by the Boyden chamber assay that the motility of MDCK cells was stimulated by HGF/SF (Stoker, 1989). We used the same system to examine the motility of



**Figure 2.** Morphology of transfected MDCK cells. HGF/SF producing clone A1 (upper), and parental MDCK cells cultured without (middle) and with 50 ng/ml recombinant human HGF/SF (lower). Cells were stained with Giemsa. Bar, 250  $\mu$ m.

HGF/SF producing MDCK cells. As shown in Table II, the motility of all four clones significantly increased when compared with parental MDCK cells.

#### **Growth Properties of HGF/SF Producing MDCK Cells**

Since exogenously added HGF/SF stimulated the growth of

**Table II. Properties of HGF/SF Producing MDCK Clones**

Clone	Concentration of HGF/SF secreted*	Number of migrated cells† (cells/mm <sup>2</sup> )	Saturation density‡ (× 10 <sup>5</sup> cells/cm <sup>2</sup> )	Number of colonies in soft agar§ (Efficiency)
	ng/ml			%
MDCK	0	70 ± 6.7	4.0	0 (0)
D4	0.6	331 ± 29.5	3.0	0 (0)
C4	1.2	329 ± 40.1	3.7	2 (0.1)
A1	3.3	389 ± 31.8	3.8	10 (0.5)
A4	6.9	215 ± 34.3	4.5	27 (1.4)

\* The cells were grown to confluence and the secreted HGF/SF in 1 ml of the medium after 24 h culture was measured by ELISA.

† Motility was assayed using Boyden chambers. Values are expressed as the mean ± SEM of two experiments performed in duplicate.

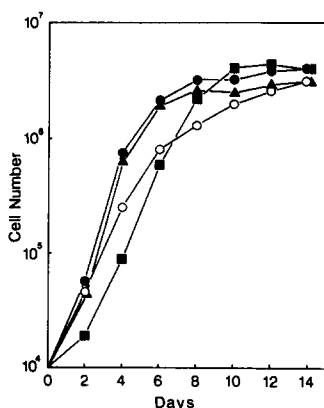
‡ The saturation density was determined by allowing cells to grow for 4 d after reaching confluence.

§ Colonies larger than 0.2 mm in diameter were counted after 28 d in culture.

MDCK cells in soft agar, the growth characteristics of the HGF/SF producing MDCK cell clones were examined. Fig. 3 shows the growth curves of these clones and parental MDCK cells on culture dish. The two clones (D4 and A1) that secrete low or average levels of HGF/SF grew at a relatively high rate when compared with the parental MDCK cells. The clone (A4) that secretes a high level of HGF/SF grew slowly at low cell density. But when the density increased, it grew at high rate. Saturation densities of these clones were similar to those of parental MDCK cells regardless of their growth rate (Table II).

The three clones (A4, A1, and C4) secreting HGF/SF at levels of 1 ng/ml or higher efficiently grew in soft agar. In contrast, growth in soft agar of the clone (D4) that secretes HGF/SF at a lower level (~0.6 ng/ml) was slow and all the colonies were smaller than 0.2 mm in diameter after 28 d in culture even though they are larger than those of parental MDCK cells. The number of colonies larger than 0.2 mm in diameter correlated roughly with the level of HGF/SF secreted (Table II).

To directly assess the effect of HGF/SF production on the *in vivo* growth properties of the cells, the HGF/SF producing clones were inoculated subcutaneously in nude mice. The clones, which acquired efficient anchorage-independent growth in soft agar (Table II), failed to induce tumor even when as many as 10<sup>7</sup> cells were inoculated. The results indicated that HGF/SF has no direct effect on the growth of MDCK cells *in vivo*.



**Figure 3.** Growth curves of HGF/SF producing clones. Cells were seeded at  $1 \times 10^4$  cells per 35-mm dish and grown in DME supplemented with 5% FBS. The cells were counted every 2 d. Each point represents the mean of two experiments performed in duplicate. (■) Clone A4; (●) clone A1; (▲) clone D4; (○) parental MDCK.

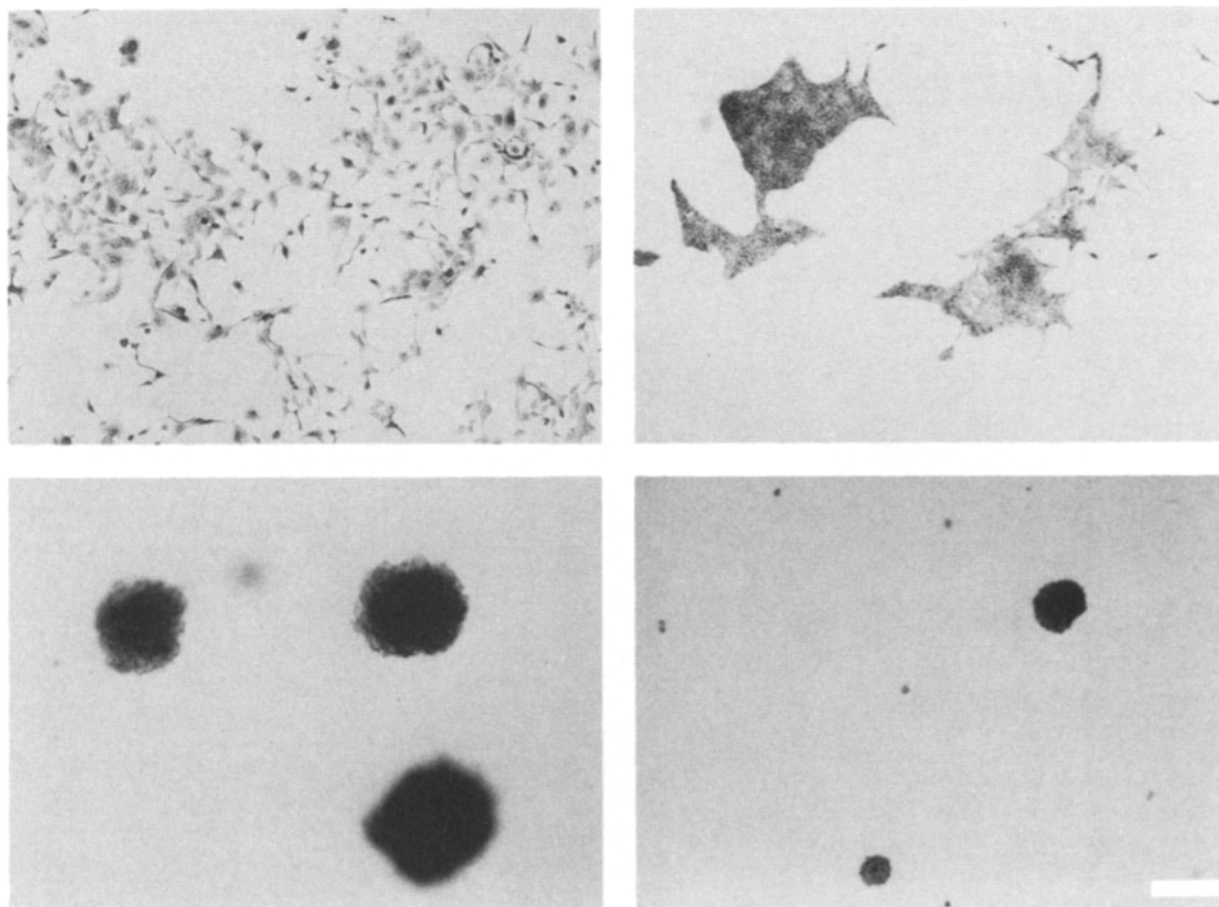
## Effect of Anti-HGF/SF Antibody on HGF/SF Producing MDCK Cells

To test whether autocrine production of HGF/SF was responsible for some of the phenotypic features of HGF/SF producing MDCK cells, we analyzed the effects of an antibody raised against human HGF/SF. In the presence of anti-HGF/SF polyclonal antibody, the HGF/SF producing MDCK cells were less well scattered (Fig. 4). In addition, the antibody inhibited growth stimulation in soft agar by exogenously added HGF/SF as well as efficient growth in soft agar of the HGF/SF producing MDCK cells. However, the antibody did not prevent growth stimulation by exogenously added EGF (Fig. 4 and Table III). The migration of HGF/SF producing MDCK cells was not prevented in the presence of the antibody (Table IV).

## Discussion

The results presented here demonstrate that human HGF/SF synthesized after transfection of MDCK epithelial cells induces changes in cell morphology from an epithelial to a fibroblastic type, cell motility and efficient anchorage-independent growth in soft agar. All the clones which secrete HGF/SF at over 0.6 ng/ml exhibited fibroblast-like scattered morphology. The scattered cells were converted to form cohesive colonies in the presence of anti-HGF/SF antibody in the culture medium. Thus, the secretion of HGF/SF was necessary for the conversion from epithelial to fibroblastic morphology. Stoker et al. (1987) have shown that HGF/SF was produced by embryo fibroblasts and by certain fibroblast lines, and that most epithelial cells were sensitive to its activity. Their results implied that HGF/SF affects mesenchymal-epithelial interactions in a paracrine fashion. Adams et al. (1991) found that ndk cells, a strain of human epidermal keratinocytes, produced HGF/SF and grew as single cells rather than as compact colonies. They also found that suramine, a polyanionic detergent, caused the ndk cells to grow in cohesive patches. From these findings, they proposed that the motile phenotype of ndk cells is due, at least in part, to autocrine production of HGF/SF. Our results showed that the fibroblastic morphology of MDCK epithelial cells was due to autocrine production of HGF/SF. It is therefore suggested that autocrine production of HGF/SF is involved in the fibroblastic phenotype. Stoker et al. (1989) demonstrated by Boyden chamber assays that fibroblasts showed high spontaneous motility and they suggested that HGF/SF is operating in these cells in an autocrine manner. However, further studies will be required to investigate whether HGF/SF production in fibroblasts influences their phenotype.

MDCK cell clones that secrete higher levels (1.2 ng/ml or higher) of HGF/SF acquired highly efficient growth properties in soft agar. This efficient growth was inhibited in the presence of anti-HGF/SF antibody, indicating that secretion of HGF/SF is required for efficient growth in soft agar. The efficient anchorage-independent growth is generally considered a property concomitant with the transformed phenotype. However, the clones that efficiently grew in soft agar did not form tumors in nude mice. Thus, they do not have properties of tumorigenic cells *in vivo*. These observations imply that the efficient growth of the cells in soft agar may not reflect the direct role of HGF/SF secretion as a growth



**Figure 4.** Conversion of phenotypes by anti-HGF/SF antibody. A HGF/SF producing clone (A4) was examined for the morphology in monolayers (*upper*) and for colony formation in soft agar (*lower*). Cells were incubated for 2 d in monolayers or for 28 d in soft agar with 0.5% anti-HGF/SF antiserum (*right*) or 0.5% normal rabbit serum (*left*). Bar, 250  $\mu$ m.

factor, but rather an indirect enhancing effect through scattering activity. Dissociated and motile cells probably have the ability to efficiently grow in semisolid medium, whereas the efficient growth of normal epithelial cells is restricted probably because of tight junctions. The number of large colonies (larger than 0.2 mm in diameter after 28 d culture) correlated roughly with the level of secreted HGF/SF, and the clone (D4) which secreted lower levels (0.6 ng/ml) of HGF/SF failed to form large colonies, even though all these clones exhibited similar scattered morphology in culture dishes. The level of HGF/SF secretion may influence scattering activity in soft agar.

**Table III.** Inhibition of Colony Formation in Soft Agar by Anti-HGF/SF Antiserum

Cells	Number of colonies in the presence of 0.5% control serum	Number of colonies in the presence of 0.5% anti-HGF/SF antiserum
MDCK (100 ng/ml HGF/SF)	35	3
MDCK (50 ng/ml EGF)	66	61
clone A1	11	2
clone A4	28	5

The motility of the HGF/SF producing MDCK cell clones was not prevented by the anti-HGF/SF antibody. Thus, the migration may be affected by intracellular action of HGF/SF. Alternatively, secreted HGF/SF may not have been completely neutralized by the antibody and the small amount of HGF/SF in the medium may affect the migration of MDCK cells.

**Table IV.** Migration of Cells in the Presence of Anti-HGF/SF Antiserum

Cells	Number of migrated cells in the presence of 0.5% control serum	Number of migrated cells in the presence of 0.5% anti-HGF/SF antiserum
MDCK		
(10 ng/ml HGF/SF)*	191 $\pm$ 20.2	53 $\pm$ 10.4
clone D4	221 $\pm$ 40.8	203 $\pm$ 47.1(247 $\pm$ 66.6)
clone C4	324 $\pm$ 17.4	298 $\pm$ 22.5(317 $\pm$ 18.4)
clone A1	353 $\pm$ 37.2	341 $\pm$ 27.6(352 $\pm$ 28.3)
clone A4	227 $\pm$ 18.5	220 $\pm$ 22.8(216 $\pm$ 24.4)

Control serum or antiserum was added to both upper and lower wells. Values are expressed as the mean  $\pm$  SEM of two experiments performed in duplicate. Cells cultured for 2 d in the presence of antiserum were used for the assay shown in parenthesis.

\* The concentration of HGF/SF required for a maximal response was 10 ng/ml. The factor was present in both wells.

The morphological change and the stimulation of anchorage-independent growth were prevented by anti-HGF/SF antibody, suggesting that the secreted HGF/SF exerts its effects through receptors on the cell surface. Little is known of HGF/SF receptor molecules on MDCK cell surface. Recently, Bottaro et al. (1991) identified a HGF/SF receptor in a human mammary epithelial cell line, whose growth is stimulated by HGF/SF, as the *c-met* proto-oncogene product. The *c-met* proto-oncogene encodes a 1408 amino acid protein with features characteristic of the tyrosine kinase family of growth factor receptors (Park et al., 1987). We have identified a HGF/SF binding 160-kD protein on the surface of MDCK cells which is probably the *c-met* proto-oncogene product (M. Komada and N. Kitamura, unpublished results). Thus, the *c-met* proto-oncogene product might be involved in the scattering of MDCK cells by HGF/SF. Further characterization of the receptor as well as the molecules involved in intracellular signal cascades will be required to understand the molecular mechanisms responsible for the action of HGF/SF on MDCK cells. The HGF/SF producing cell clones which we have established in this study will be useful for such studies.

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