Hepatocyte growth factor is an invasion/migration factor of rat urothelial carcinoma cells in vitro

Tetsuya Tamatani, Kazunori Hattori, Anand Iyer, Kanako Tamatani and Ryoichi Oyasu

Department of Pathology, Northwestern University Medical School, Chicago, IL 60611-3008, USA

1To whom correspondence should be addressed
Email: r-oyasu@nwu.edu

Hepatocyte growth factor (HGF) plays an important role in the growth, progression and angiogenesis of various tumors. It is reported that patients with urinary bladder cancer have elevated levels of HGF in urine and that bladder cancer tissue contains an increased amount of HGF. Thus, the data suggest a functional role of HGF in urinary bladder cancer. We evaluated the mechanistic role of HGF in urinary bladder carcinoma in vitro using the rat urothelial cell lines MYP3 (anchorage-dependent and non-tumorigenic in athymic nude mice), LMC19, MYU3L, T6 and AS-HTB1 (anchorage-independent and tumorigenic). The HGF receptor c-met was expressed by all of the cell lines, as determined by northern blot. In MYP3 cells, HGF strongly stimulated anchorage-dependent growth, but not migration, invasion or secretion of matrix metalloproteinases (MMPs). In LMC19, T6 and AS-HTB1 cells, HGF stimulated migration, invasion and secretion of MMPs. Anchorage-dependent growth stimulation was limited to AS-HTB1 cells. MYU3L cells were refractory to HGF in both growth and invasion assays. However, a neutralizing antibody and an anti-sense oligonucleotide to HGF partially inhibited the growth only of MYU3L cells, the finding being indicative of an autocrine stimulatory mechanism. HGF mRNA expression and protein synthesis were induced in bladder stromal cells by the conditioned medium of carcinoma cell lines, and IL-1β and basic fibroblast growth factor were identified as cancer cell-derived HGF-releasing factors. These results suggest that HGF acts as a mitogen in a non-tumorigenic cell line, whereas in tumorigenic cell lines it acts as an invasion and migration factor by either a paracrine or an autocrine mechanism.

Introduction

Hepatocyte growth factor (HGF) was first identified as a stromal cell-derived cytokine, which is mitogenic to hepatocytes (1–3), and subsequently has been demonstrated to be a mitogen, motogen, morphogen and angiogenic factor in vitro of normal and neoplastic epithelial cells (4–6). It may also play a critical role in biological processes including wound healing, development and carcinogenesis (7). HGF protein is synthesized by fibroblasts and smooth muscle cells (8,9). By immunohistochemical study, HGF has been demonstrated in various human organs, including the respiratory tract, breast, digestive tract, kidney and urinary bladder (10).

The HGF receptor, which belongs to the family of receptors of tyrosine kinases, is encoded by the c-met proto-oncogene (11). c-Met receptor is expressed by epithelial cells of various organs, including the liver, digestive tract, kidney, urinary bladder and lung (12,13). In the urinary bladder, c-met is expressed in urothelial cells, endothelial cells and smooth muscle cells (13).

HGF can stimulate cell motility, as well as invasion and secretion of proteinases which destroy the basement membrane (14,15). Therefore, it may play an important role in tumor invasion and progression, and HGF synthesis by the mesenchymal cells may be critical for the migration and invasion of tumor cells.

HGF is detected in urine, and patients with transitional cell carcinoma of the bladder, especially with muscle-invasive carcinoma, have higher urinary levels of HGF than do normal subjects. Also, bladder cancer tissue contains an increased amount of HGF (13,16). Although, a causal role of HGF has been suggested in the progression of bladder cancer (13,16), its precise role remains unknown.

To evaluate the mechanistic role of HGF in bladder tumor growth, we examined the effect of HGF in vitro on the growth, migration, invasion of bladder carcinoma cells, and the effect of carcinoma cells on the HGF mRNA and protein synthesis by stromal cells. We used rat urothelial carcinoma cell lines and rat stromal cells that have been established in our laboratory. Our results indicate that HGF synthesis and release by stromal cells is closely controlled by carcinoma cells and that HGF plays a critical role in the invasion of bladder cancer by paracrine and also occasionally by autocrine mechanisms.

Materials and methods

Cells and cell culture

All of the cell lines (MYP3, LMC19 and MYU3L) and MYP3 derivatives (MYP3, T6 and AS-HTB1) used were developed in our laboratory. MYP3 was established from a small nodule that was found in a heterotopically transplanted urinary bladder (17) after treatment in vivo with N-methyl-N-nitrosourea (MNU). MYP3 is anchorage-dependent for growth and is non-tumorigenic in athymic nude mice (18). T6 is a bladder carcinoma cell line established from MYP3 cells after treatment in vitro with 200 μg/ml of MNU once a week for 4 consecutive weeks. T6 cells formed a poorly differentiated transitional cell carcinoma in athymic nude mice (19). The AS-HTB1 cell line was isolated from a poorly differentiated transitional cell carcinoma that developed in an athymic nude mouse after s.c. inoculation of MYP3 cells transfected with antisense p53 cDNA (20). LMC19 was established from a pulmonary metastasis in a nude mouse after s.c. inoculation of rat carcinoma cells of clonal origin (21). It is tumorigenic, highly invasive and also metastatic. MYU3L is a rat urinary bladder carcinoma cell line that is tumorigenic and highly invasive, but non-metastatic (18). A primary culture of stromal cells was established from the submucosa of a normal rat bladder.

MYP3, T6 and AS-HTB1 cells were grown in Ham’s F-12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10 μM non-essential amino acids (Gibco BRL), 2.7 mg/ml dextrose (Sigma, St Louis, MO), 1 μg/ml of hydrocortisone (Sigma), 5 μg/ml of transferrin (Gibco BRL), 10 μg/ml of...
insulin (Gibco BRL), 10 µg/ml of epidermal growth factor (Gibco BRL), 100 µg/ml of streptomycin and 100 U/ml of penicillin (Gibco BRL). When supplemented with 10% fetal bovine serum (FBS; Gibco BRL), the medium was designated as complete medium. LMC19 and MYU3L cells were grown in Ham’s F-12 medium supplemented with 5% FBS, 10 µM non-essential amino acids, 100 µg/ml of streptomycin and 100 U/ml of penicillin. Stromal cells were grown in DMEM (Gibco BRL) supplemented with 10% FBS, 10 µM non-essential amino acids, 100 µg/ml streptomycin and 100 U/ml of penicillin. All cultures were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

**Cell growth assay**

Cells were grown on a flat-bottomed 96-well plate at 1×10⁴ cells/well containing the complete medium or F-12 medium with 5% FBS. Twenty-four hours later, cells were grown in F-12 medium with (MYP3, T6, AS-HTB1) or without (LMC19, MYU3L) 0.1% FBS and with human recombinant HGF (0.1–10 ng/ml) (a gift from Dr T. Nakamura) (22). After 3 days of culture, cell proliferation was assayed by addition of 20 µg of the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 1 mg/ml; Sigma). The blue dye taken up by cells during 4 h culture was dissolved in dimethyl sulfoxide (100 µl/well), and its optical density at 490 nm was read on an automated microplate reader (Bio-Tek, Winooski, VT). A preliminary study by MTT assay showed that absorbance was directly proportional to the number of cells.

**Growth in soft agar**

Cells (5×10³ MYP3, T6 and AS-HTB1 cells, and 1×10⁴ LMC19 and MYU3L cells) suspended in 2 ml of 0.3% Noble agar (Difco, Detroit, MI) in the presence of HGF (10 ng/ml) were layered over 2 ml of 0.6% agar in the complete medium in 35 mm dishes (Falcon) for MYP3, T6 and AS-HTB1 cells or F-12 medium with 5% FBS in 35 mm dishes for LMC19 and MYU3L cells. One milliliter of 0.3% agar in the complete medium containing HGF was added on days 9 and 18. After 21 or 28 days of culture, colonies of >20 cells were counted.

**Isolation of cytoplasmic RNA and northern blot analysis**

When cells grown in monolayer reached an early phase of confluency, RNA was prepared by lysing of cells in a hypotonic buffer containing NP-40 (Sigma), followed by removal of nuclei. Cytoplasmic RNA was subjected to northern blot analysis and semiquantitative reverse transcription–polymerase chain reaction (RT–PCR).

For northern blot analysis, cytoplasmic RNA (20 µg) was electrophoresed onto a formaldehyde–1.0% agarose gel and blotted onto a nylon filter with a GeneScreen Plus (New England Nuclear, Boston, MA)–coated surface of the inner cup. The probes used were an upstream and downstream primer, respectively, for GAPDH (Perkin Elmer, Norwalk, CT) under the following conditions: 94, 55 and 72°C for 60 s each; 35 cycles for IL-6, bFGF and PDGF-A and 25 cycles for IL-1α. The filters were hybridized with a 32P-labeled CDNA probe in 50% formamide, 5× saline–sodium phosphate–EDTA, 0.1% SDS, 5× Denhardt’s solution and 100 µg/ml of salmon sperm DNA (Sigma) at 65°C for 12–16 h. The probes used were an XhoI/XhoI fragment of pHF10 (10 µg) for the c-met proto-oncogene and an XhoI/XhoI fragment of pHF2aβ (10 µg) for the translation–initiation region of rat β-actin (American Type Culture Collection, Rockville, MD).

**In vitro migration assay**

Cell migration was assayed in a modified Boyden chamber (Nuroprobe, Santa Clara, CA) with a polyvinyl pyrrolidone-free polycarbonate filter with 3-µm porosity (Nucleopore, Pleasanton, CA). The filters were precoated with a T.Tamatani (0.1–10 ng/ml) (data not shown). Additionally, a 20 base sense (5′-GACAAGC-TGAAGAGGGGAC-3′) and an anti-sense (5′-GTTCCCCACACATGTTTGTGACCTTGAC-3′) oligonucleotide (10 µM) for the translation–initiation region of rat HGF cDNA were added to the cultures for assessment of their effect on the anchorage-dependent growth of cells. After 3 days in culture, cells were counted by MTT assay.

**Preparation of CM**

Carcinoma cells were grown to confluence in a 100 mm dish, washed 3 times with serum-free medium and cultured for an additional 72 h in F-12 medium containing 1% FBS. The medium was collected and clarified by centrifugation at 3000 g.

**Induction of HGF mRNA by stromal cells and expression of HGF-inducible factors by bladder carcinoma cells**

Stromal cells (2.0×10⁶ cells/dish) were treated for 12 h in the presence or absence of carcinoma cell-derived CM added at 1:1 (v/v), and RNA was extracted from cells. Expression of HGF and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA, which was used as an internal control, was assessed by a semiquantitative RT–PCR method. Cytoplasmic RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Gibco BRL) at 42°C for 60 min in 20 µl mixture with random hexamer primers (Gibco BRL). One microliter of reverse-transcribed mixture was used for the PCR. The following primers were used as a downstream and upstream primer, respectively, for GAPDH

- 5′-GACAAGC-TGAAGAGGGGAC-3′ and 3′-CAATGTCGCTGAGGTTTGGCTAGACAC-3′ as an upstream and a downstream primer, respectively, for GAPDH, and
- 5′-GGAACTCATCCATACCTCTCAGG-3′ and 5′-CAATGTCGCTGAGGTTTGGCTAGACAC-3′ as an upstream and a downstream primer, respectively, for GAPDH.

To look for the carcinoma cell-derived factor(s) which stimulate(s) HGF release from stromal cells, we examined the expression of Interleukin (IL)-1α, IL-1β, IL-6, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)-A mRNA by semiquantitative RT–PCR. The reaction was carried out at 94, 60 and 72°C for 60 s each; 35 cycles for IL-1β, IL-6, bFGF and PDGF-A and 25 cycles for IL-1α. Primers used as upstream and a downstream primers, respectively, were 5′-CTTGAGAATCTTCCGCTC-3′ and 5′-CTTGAGAATCTTCCGCTC-3′; 5′-CACTGTCGCTGAGGTTTGGCTAGACAC-3′ and 5′-CAATGTCGCTGAGGTTTGGCTAGACAC-3′ for IL-1α; 5′-GACAAGC-TGAAGAGGGGAC-3′ and 5′-CTTGAGAATCTTCCGCTC-3′. The primers used were 5′-GACAAGC-TGAAGAGGGGAC-3′ and 5′-CTTGAGAATCTTCCGCTC-3′ for PDGF-A. Amplified cDNA was electrophoresed on a 1.5% agarose gel, and the gel was stained with 100 µg/ml ethidium bromide.

**Measurement of HGF in culture media**

We measured the concentration of HGF in CM of the carcinoma and stromal cells. Stromal cells were seeded on 6-well plates at 4×10⁵ cells/well and were cultured for 24 h in the presence or absence of carcinoma cell-derived CM added at 1:1 (v/v) with 2 U/ml heparin (Sigma), and HGF in CM was assayed by HGF EIA kit (Institute of Immunology, Tokyo, Japan).
Treatment with exogenous HGF significantly stimulated invasion by T6 and AS-HTB1 cells, whereas HGF was inhibitory to MYP3 and MYU3L cells (Table I). T6 and AS-HTB1 cells showed little invasive-ness (1.6 and 5.4 cells, respectively). On the other hand, migration of LMC19, T6 and AS-HTB1 cells was stimulated strikingly by HGF (17-, 18- and 24-fold increase over the respective control; \( P < 0.01 \), for each comparison). MYU3L cells migrated actively (285.7 cells per 10 fields/well), and HGF (10 ng/ml) had no effect. AS-HTB1 cells migrated actively (285.7 ± 80.5 cells), but HGF had no effect (307.7 ± 56.9 cells).

### Effect of HGF on invasion

LMC19 and MYU3L cells were highly invasive (95.4 ± 12.3 and 91.4 ± 12.1 cells/five fields, respectively) (Table III), whereas MYP3, T6 and AS-HTB1 cells showed little invasiveness (1.6 ± 0.4, 6.4 ± 1.6 and 5.4 ± 1.7 cells, respectively). HGF significantly stimulated invasion by T6 and AS-HTB1 cells, resulting in a 3- and 21-fold increase over the untreated controls (\( P < 0.01 \) for each comparison). In contrast, the effect of HGF was less on LMC19 (142.2 ± 6.5 cells; \( P < 0.05 \)). HGF had no effect on MYP3 or MYU3L cells.

### Effect of HGF on secretion of MMPs and urokinase-type plasminogen activator

All carcinoma cell lines secreted MMP9 into medium and, with the exception of MYU3L, all carcinoma cell lines also secreted MMP2. HGF treatment stimulated MMP9 activity significantly in all carcinoma cell lines. MMP2 activity, however, was not affected in any of the cell lines. In addition to

### Results

#### Expression of c-met mRNA

By northern blot analysis, c-met mRNA was detected in all of the cell lines (Figure 1) and there was no significant difference in the expression level among cell lines. The expression level of c-met mRNA was dependent on the concentration of growth factors.

#### Effect of HGF on anchorage-independent growth

Exogenous HGF stimulated the growth of MYU3L and LMC19 cells in a dose-dependent manner (at 1 and 10 ng/ml; \( P < 0.05 \) for each comparison). Neither AS-HTB1 nor T6 cells showed a significant response to HGF, whereas HGF was inhibitory to MYU3L cells (Figure 2).

In the anchorage-independent growth assay, LMC19 and T6 colonies increased significantly (162 and 393% of the respective control, \( P < 0.05 \) for each comparison), whereas the response of AS-HTB1 cells was marginal (122% of control), and MYU3L cells did not respond at all (Table I).

### Table I. Effect of HGF on anchorage-independent growth

<table>
<thead>
<tr>
<th>Cells</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HGF absent</td>
</tr>
<tr>
<td>MYP3</td>
<td>0</td>
</tr>
<tr>
<td>LMC19</td>
<td>164.0 ± 9.9</td>
</tr>
<tr>
<td>MYU3L</td>
<td>184.3 ± 9.0</td>
</tr>
<tr>
<td>T6</td>
<td>25.5 ± 6.7</td>
</tr>
<tr>
<td>AS-HTB1</td>
<td>50.5 ± 4.1</td>
</tr>
</tbody>
</table>

Cells (5x10^3 of MYP3, T6 and AS-HTB1 cell lines, and 1x10^3 of LMC19 and MYU3L cell lines) were cultured in soft agar medium with or without HGF (10 ng/ml). After 21 (LMC19 and MYU3L) or 28 days (MYP3, T6 and AS-HTB1), colonies consisting of >20 cells were counted. *\( P < 0.05 \), as compared with respective untreated controls.

#### Effect of HGF on migration

We tested the effect of exogenous HGF on the migration of cells by using the modified Boyden chamber (Table II). MYP3 cells were the least migratory among the cell lines (5.0 ± 2.2 cells per 10 fields/well), and HGF (10 ng/ml) had no effect. On the other hand, migration of LMC19, T6 and AS-HTB1 cells was stimulated strikingly by HGF (17-, 18- and 24-fold increase over the respective control; \( P < 0.01 \), for each comparison). MYU3L cells migrated actively (285.7 ± 80.5 cells), but HGF had no effect (307.7 ± 56.9 cells).

### Table II. Effect of HGF on migration of cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Migrated cells/10 fields per well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HGF absent</td>
</tr>
<tr>
<td>MYP3</td>
<td>5.0 ± 3.2</td>
</tr>
<tr>
<td>LMC19</td>
<td>25.8 ± 7.8</td>
</tr>
<tr>
<td>MYU3L</td>
<td>285.7 ± 80.5</td>
</tr>
<tr>
<td>T6</td>
<td>4.0 ± 1.8</td>
</tr>
<tr>
<td>AS-HTB1</td>
<td>13.1 ± 7.7</td>
</tr>
</tbody>
</table>

Cells (4.2x10^3 cells) were cultured with or without HGF (10 ng/ml) for 4 h. Filters were stained with hematoxylin, and cells migrating through the filter were counted per 10 high-power fields. *\( P < 0.01 \), as compared with respective untreated controls.

#### Effect of HGF on secretion of metalloproteinase (MMP) and urokinase-type plasminogen activator

All carcinoma cell lines secreted MMP9 into medium and, with the exception of MYU3L, all carcinoma cell lines also secreted MMP2. HGF treatment stimulated MMP9 activity significantly in all carcinoma cell lines. MMP2 activity, however, was not affected in any of the cell lines. In addition to

### Table III.

#### Expression of c-met mRNA

<table>
<thead>
<tr>
<th>Cells</th>
<th>c-met mRNA</th>
<th>β-actin mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYU3L</td>
<td>Lane a</td>
<td>Lane c</td>
</tr>
<tr>
<td>MYU3L</td>
<td>Lane b</td>
<td>Lane c</td>
</tr>
<tr>
<td>LMC19</td>
<td>Lane a</td>
<td>Lane c</td>
</tr>
<tr>
<td>LMC19</td>
<td>Lane b</td>
<td>Lane c</td>
</tr>
</tbody>
</table>

To neutralize any IL-1β and bFGF that might be secreted by carcinoma cells, polyclonal goat anti-IL-1β antibody (R&D), and monoclonal mouse anti-bovine bFGF antibody (Upstate Biotechnology, Lake Placid, NY) at a final concentration of 5 and 25 µg/ml, respectively, were added to stromal cell cultures. For the control cultures, non-immune IgG at the same concentrations was added.

### Statistical analysis

The effect of anti-HGF antibody and anti-sense oligonucleotide was tested by one-way analysis of variance (Fisher’s protected least significant difference). Other comparisons (MTT assay, soft agar growth, migration and invasion assays, and measurement of HGF) were made with Mann–Whitney U test. A \( P \)-value <0.05 was considered to indicate significance.

### Effect of HGF on rat urothelial cells

To further investigate the effect of HGF on cell proliferation, we cultured rat urothelial cells in the presence or absence of HGF for 4 h. The cells were then stained with hematoxylin and the number of cells migrating through the filter was counted. As shown in Figure 2, HGF significantly stimulated the growth of rat urothelial cells (Fig. 2).
Table III. Effect of HGF on invasion of cells through Matrigel-coated membrane

<table>
<thead>
<tr>
<th>Cells</th>
<th>Invading cells/5 fields</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HGF absent</td>
</tr>
<tr>
<td>MYP3</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>LMC19</td>
<td>95.4 ± 12.3</td>
</tr>
<tr>
<td>MYU3L</td>
<td>91.4 ± 12.1</td>
</tr>
<tr>
<td>T6</td>
<td>6.4 ± 1.6</td>
</tr>
<tr>
<td>AS-HTB1</td>
<td>5.4 ± 1.7</td>
</tr>
</tbody>
</table>

Cell suspension, 200 µl (5.0 × 10⁵ cells/ml) in F-12 with 1% FBS, was placed on the Matrigel-coated surface of the inner cup. F-12 containing 1% FBS with or without HGF (10 ng/ml) was added to the outer compartment. After 72 h incubation, cells that invaded through the Matrigel-coated membrane were stained with hematoxylin and counted under the microscope. Results are expressed as average number of cells present in 5 fields per membrane. *P < 0.05, compared with untreated control; **P < 0.01, as compared with respective untreated control.

MMP9, MYU3L also secreted MMP1, and its activity was enhanced by HGF treatment. MYP3 showed a trace MMP2 and MMP9 activity, and HGF had no effect (Figure 3).

All types of cells demonstrated plasminogen activator activity of the urokinase type, but there was no effect of HGF on the enzyme activity (data not shown).

Effect of anti-HGF antibody and anti-sense HGF oligonucleotide on anchorage-dependent growth

The growth of MYP3, T6, LMC19 and AS-HTB1 cells was not affected at all by anti-human HGF neutralizing antibody, whereas the growth of MYU3L was slightly, but significantly inhibited (86% of the untreated control; P < 0.05; Figure 4A). The mild response could be due to the use of an antibody against human rather than rat HGF. Addition of the anti-sense oligonucleotide suppressed the growth of MYU3L cells to a greater extent (57% of the untreated control; P < 0.005; Figure 4B).

Release of HGF by MYP3 and carcinoma cells, and stromal cell induction of HGF mRNA and protein stimulated by carcinoma cell-derived CM

HGF protein was not detected in CM of MYP3, LMC19, T6 and AS-HTB1 cells. Only MYU3L cells secreted 1.1 ± 0.29 ng/ml HGF protein.

We examined the effect of carcinoma cell-derived CM on stromal cell induction of HGF mRNA (Figure 5) and protein (Figure 6). Untreated stromal cells did not express HGF mRNA (Figure 5, lane a). HGF expression at mRNA and protein levels was stimulated by the CM of all types of epithelial cells. However, the release was at much higher levels after treatment with CM derived carcinoma cell cultures (LMC19, MYU3L, T6 and AS-HTB1) than that after treatment with CM derived from MYP3 cells (P < 0.05 for each comparison; Figure 6).

Identification of carcinoma cell-derived inducers of HGF

To determine which factor(s) derived from carcinoma cells is (are) responsible for stromal cell induction of HGF, we examined the expression of IL-1α, IL-1β, IL-6, bFGF and PDGF-A mRNA, most of which are reportedly inducers of HGF (9,25). Under the experimental conditions used, IL-1β mRNA was expressed by T6 and MYP3 cells and at a much higher level by the former. bFGF mRNA was expressed by all cancer cell lines, but at much higher levels by LMC19, AS-HTB1 and MYU3-L cells than others (Figure 7). IL-6 mRNA was not detected, and no significant difference was observed in IL-1α and PDGF-A expression among the cell lines (data not shown).

Next, we examined if these factors act as inducers of HGF. Addition of anti-IL-1β antibody inhibited the HGF production only of stromal cells treated with T6 CM (65% of the control; P < 0.05; Figure 6). On the other hand, anti-bFGF antibody effectively reduced HGF production of stromal cells treated

**T.Tamatani et al.**
as compared with the untreated control; * \( P < 0.05 \) as compared with the untreated control; † \( P < 0.05 \) as compared with the non-immune IgG control; ‡ \( P < 0.05 \) as compared with the non-tumorigenic cell line MYP3.

Fig. 6. Induction of HGF protein by rat stromal cells stimulated by carcinoma cell-derived CM. Cells were cultured for 24 h in the presence or absence of carcinoma cell-derived CM added at 1:1 (v:v). Neutralizing anti-IL-1β and anti-bFGF antibodies at final concentrations of 5 and 25 μg/ml, respectively, were added. For the controls non-immune IgG at the same concentrations was used. Bars denote SD of triplicate samples. + \( P < 0.05 \) as compared with the untreated control; † \( P < 0.05 \) as compared with the non-immune IgG control; ‡ \( P < 0.05 \) as compared with the non-tumorigenic cell line MYP3.

Fig. 7. IL-1β and bFGF mRNA expression of carcinoma cells by RT-PCR. Five micrograms of cytoplasmic RNA and primers specific for rat IL-1β, bFGF and GAPDH were used. cDNA fragments generated are of 520, 464 and 718 bp, respectively. Lane a, MYP3; lane b, LMC19; lane c, MYU3L; lane d, AS-HTB1; lane e, T6.

with LMC19, MYU3L and AS-HTB1 CM (66, 56 and 54%, respectively, of controls; \( P < 0.05 \) for each comparison).

Discussion

The growth, invasion and metastatic potential of tumor cells are greatly influenced by their interaction with stromal fibroblasts (25). A cooperative interaction between cancer cells and the underlying stromal cells has been demonstrated in in vitro as well as in in vivo studies (8,16,26). Using carcinoma cell lines, Nakamura and colleagues (25,26) demonstrated that HGF produced by stromal cells is a potent growth factor that stimulates migration and invasion of carcinoma cells, and that HGF synthesis by stromal cells is mediated by the growth factors released by cancer cells. The HGF inducers were shown to be IL-1α, IL-1β, bFGF, PDGF and TNF-α (9).

In urinary bladder carcinoma, Rosen and colleagues (13) have demonstrated a clear association of HGF synthesis by tumor with its release into urine, and cancer cell-mediated HGF synthesis by stromal cells was suggested by an in vitro assay (10).

The present investigation critically evaluated the mechanistic role of HGF in bladder cancer growth and invasion. Several important findings were observed. First, non-tumorigenic as well as tumorigenic rat bladder epithelial cells do not release HGF (except for one carcinoma cell line, MYU3L), but express HGF receptor c-met. Secondly, all cancer cell lines except for MYU3L respond to exogenous HGF with marked acceleration in migration and invasion associated with an increase in MMP activity. The response to HGF was most striking in LMC19 and AS-HTB1 cells. Such a response was not observed in the non-tumorigenic cell line MYP3, despite the presence of HGF receptor. Thirdly, the proliferative response to HGF was best demonstrated by MYP3 cells, and was absent to modest at best with cancer cell lines. Thus, the action of HGF as a mitogen was clearly independent of its action as an invasion factor. This difference in HGF action has been suggested to be due to a difference in the signal transduction mechanism after phosphorylation of tyrosine residues in the c-met protein (27). Fourthly, carcinoma cell-derived CM stimulated HGF synthesis and release by stromal cells at levels much higher than that after stimulation by non-tumorigenic MYP3-derived CM (\( P < 0.05 \)). We identified IL-1β and bFGF as carcinoma cell-derived inducers of HGF.

Finally, among the cancer cell lines examined, MYU3L differed considerably from the others in response to HGF despite the presence of c-met, and anchorage-dependent growth was inhibited with increasing concentrations of HGF. In order to test the possibility that the refractoriness was due to an established autocrine growth-stimulatory mechanism, we tested cells with anti-HGF antibody and anti-sense HGF oligonucleotide. Nearly 50% inhibition was observed in the anchorage-dependent growth. Such an inhibitory effect was not shown by any other cell lines. MYU3L cells produced HGF protein, while other cells did not. The finding establishes that in MYU3L HGF acts as an autocrine growth factor, whereas in other cell lines HGF operates as a paracrine factor.

From our observation described above, we can conclude that epithelial stromal interactions exist between urothelial cell and stromal cells (Figure 8). In benign and perhaps also low-grade bladder carcinoma, HGF is released at a low level by stromal cells in response to epithelial cell-derived factor(s), and acts as a mitogen to urothelial cells. In high-grade invasive carcinoma, HGF released from stromal cells in response to cancer cell-derived inducers acts as a migration and invasion factor to carcinoma cells. In exceptional cases, HGF may stimulate cancer cell growth by an autocrine mechanism, as was also shown by Rahimi et al. (28). Our observations are very much in keeping with the findings by Joseph et al. (13) and Rosen et al. (16) that bladder cancers especially those of high-grade invasive tumors contain HGF at high levels. Our data offer mechanistic support for the conclusion that HGF is involved in the invasive behavior of high-grade invasive bladder cancer.
T. Tamatani et al.

Acknowledgements

This study was supported by NIH grant CA14649 and the Joseph L. Mayberry Senior Endowment Research Fund.

References


Received August 6, 1998; revised February 12, 1999; accepted March 1, 1999