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# Acquisition of Invasive Phenotype in Gallbladder Cancer Cells via Mutual Interaction of Stromal Fibroblasts and Cancer Cells as Mediated by Hepatocyte Growth Factor

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**Growth and motility of carcinoma cells are regulated through their interactions with host stromal cells, i. e., tumor-stromal interactions. Hepatocyte growth factor (HGF), a ligand for c-Met tyrosine kinase, is a stromal-derived regulator of growth, motility, and morphogenesis. HGF stimulated proliferation and motility of GB-d1 gallbladder carcinoma cells from a patient with gallbladder cancer. HGF induced *in vitro* invasion of GB-d1 cells into a collagen gel matrix, and this potent, invasive effect was not seen with epidermal growth factor, transforming growth factor-β1, basic fibroblast growth factor, or platelet-derived growth factor. Although GB-d1 did not produce HGF, the cells did produce a factor which enhances HGF production in human skin fibroblasts, and this factor proved to be interleukin-1β (IL-1β). When GB-d1 cells were co-cultured with fibroblasts such that a collagen gel matrix was layered between the GB-d1 cells and fibroblasts, GB-d1 cells invaded the gel, but invasion of the cells in the co-culture system was inhibited by antibodies against HGF and partially inhibited by antibodies against IL-1β. Thus, GB-d1 cell-derived IL-1β stimulates HGF production in stromal fibroblasts and HGF up-regulated in the fibroblasts induces invasion of GB-d1 cells. The looped interaction of carcinoma cells and stromal fibroblasts mediated by HGF and a HGF-inducer such as IL-1β may be one mechanism which would explain the acquisition of malignant phenotype through tumor-stromal interactions.**

Key words: Gallbladder cancer — Hepatocyte growth factor — Tumor invasion — Tumor-stromal interactions

Interactions between epithelium and mesenchyme mediate crucial aspects of normal development, affecting tissue induction, organogenesis, cell movement, and morphogenesis of specific multicellular structures.[1–3] Epithelial-mesenchymal (or -stromal in adult stages) interactions are also considered to be important in regeneration, morphogenesis and functional differentiation of epithelial tissues.[3–5] A similar conceptual framework is likely to be applicable to specific interactions between carcinoma cells (tumor cells originating from epithelial cells) and host stromal cells. *In vivo* growth of carcinoma cells could be markedly accelerated by a broad spectrum of fibroblasts[6,7] and *in vitro* invasion of oral squamous cell carcinoma cells was induced by co-cultivation with stromal fibroblasts.[8] These findings led to the notion that growth, invasion and metastatic potentials of tumor cells are influenced as a result of interaction with normal stromal fibroblasts, and the molecular mechanisms underlying these tumor-stromal interactions have received much attention.

Hepatocyte growth factor (HGF), originally characterized as a potent mitogen for mature hepatocytes, is a mesenchymal- (or stromal-) derived multipotent growth factor which predominantly targets a wide variety of epithelial, carcinoma, and endothelial cells.[5,9,10] In addition to mitogenic activity, HGF elicits a unique morphogenic activity, inducing branching tubulogenesis in several lines of epithelial cells.[11–14] HGF is also an angiogenic factor *in vitro* and *in vivo*.[15–17] Scatter factor, originally identified as fibroblast-derived motility factor for epithelial cells,[18] proved to be the same molecule as HGF,[19–21] and this finding implied a potent motogenic potential (enhancement of cell motility) for a wide variety of cells, including carcinoma cells. Based on these mitogenic, motogenic and morphogenic activities and the preferential respective expression of HGF and its receptor in mesenchymal (or stromal) and epithelial tissues,[5,22] HGF is considered to be a mediator in epithelial-mesenchymal interactions during both organogenesis and organ regeneration.

HGF is a heterodimeric molecule composed of a four kringle-containing α-chain and serine protease-like β-chain.[23,24] This growth factor has structural homology with a five kringle-containing serine protease, plasminogen, but has no proteolytic activity due to replacement of amino acids essential for protease catalytic activity.[24] HGF binds to the c-met protooncogene product of heterodimeric tyrosine kinase, composed of a 50 kDa α-

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chain and a 145 kDa $\beta$-chain.[25,26] The c-Met/HGF receptor is structurally distinct from other receptor-type tyrosine kinases, and the related receptor tyrosine kinases, Ron and Sea are members of the Met/HGF receptor family.[27,28]

On the basis of the current notion that HGF is an important mediator in epithelial-mesenchymal interactions, affecting cell growth, morphogenesis, and cell motility, we asked whether HGF and regulatory factors for the expression of HGF might play roles in tumor-stromal interactions. We now report that HGF induces mitogenesis and invasion of gallbladder carcinoma cells, as a fibroblast-derived humoral factor, while carcinoma cells secrete an inducer of HGF production in fibroblasts. Looped interaction between carcinoma cells and fibroblasts mediated by HGF and its inducer may possibly be involved in acquisition of malignant phenotypes in gallbladder carcinoma cells, through tumor-stromal interactions.

## MATERIALS AND METHODS

**Cell culture** The human gallbladder carcinoma cell line, GB-d1 was initially established from poorly differentiated adenocarcinoma of the gallbladder, as described elsewhere.[29] GB-d1 cells were routinely cultured in William's medium E (WE) supplemented with 100 $\mu$g/ml kanamycin and 10% fetal calf serum (FCS). GB-d1 cells can also be cultivated in Dulbecco's modified Eagle's (DME) medium supplemented with kanamycin and FCS, the growth rate being similar to that when WE medium is used.

Normal human skin fibroblasts were initially allowed to proliferate outward from dermal tissue obtained during plastic surgery and the cells were collected and cultured in DME medium supplemented with 10% FCS.

**Growth factors and antibodies** Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells transfected with expression plasmid for human HGF cDNA.[24,30] HGF used in this study was of the 5-amino-acids-deleted type and its purity exceeded 98%, as determined by SDS-polyacrylamide gel electrophoresis and protein staining. Human recombinant epidermal growth factor (EGF) was a kind gift from Earth Pharmaceutical Company (Akoh). Human transforming growth factor-$\beta$1 (TGF-$\beta$1) was purified from human platelets, as described elsewhere.[31] Human recombinant platelet-derived growth factor (PDGF) and bovine recombinant basic fibroblast growth factor (bFGF) were obtained from Toyobo Co. (Osaka), and human recombinant interleukin-1$\alpha$ (IL-1$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$) were obtained from Genzyme Co. (Boston). Polyclonal antibodies against IL-1$\beta$ and IL-1$\alpha$, and monoclonal anti-human EGF receptor antibody were obtained from Genzyme Co. (Boston). Polyclonal anti-PDGF antibody was obtained from R & D Systems (Minneapolis) and monoclonal anti-bFGF was a kind gift from Dr. K. Nishikawa (Kanazawa Medical College).

Polyclonal antibody against human HGF was prepared from the serum of a rabbit immunized with human recombinant HGF. IgG was purified by using protein A-Sepharose (Pharmacia, Uppsala) and anti-human HGF IgG (1 $\mu$g/ml) completely neutralized the biological activities of 1 ng/ml human HGF.

***In vitro* invasion assay** Seven volumes of type 1 collagen solution (CELLGEN, Type I-AC, Koken Co., Tokyo) were mixed with two volumes of $\times 5$ concentration of WE medium and one volume of FCS, under ice-cooling. The preparation was added to the inner cup of a Trans-well chamber (Costar, Cambridge, MA) and gelled at 37°C in a humidified incubator. GB-d1 cells were plated on collagen gel at a density of $10^5$ cells/cm$^2$ and cultured for 15 h. The upper and lower media were changed to fresh medium supplemented with 5% FCS and growth factors were added to the lower medium, so that growth factors could penetrate the collagen gel and reach the cells. The upper and lower media were changed every three days and the cells were cultured for 12 days.

For the co-cultivation of GB-d1 cells and fibroblasts, human dermal fibroblasts were initially plated in the inner cup of a Trans-well chamber with attached collagen film (Costar) at a density of $5\times10^4$ cells/cm$^2$ and cultured for 24 h. The medium was aspirated, then collagen solution was overlayed and gelled as described above. GB-d1 cells were suspended in DME medium containing 5% FCS and plated on collagen gel at a density of $10^5$ cells/cm$^2$. The medium was changed every three days and the cells were cultured in DME medium supplemented with 5% FCS for 12 days. When the cells were cultured in the presence of antibodies, preimmune IgG, anti-human HGF IgG, or anti-IL-1$\beta$ IgG was added at the final concentration of 10 $\mu$g/ml.

**Measurement of HGF production in fibroblasts** Human dermal fibroblasts were seeded on a 48-well plate (Costar) at a density of $5\times10^4$ cells/cm$^2$ and cultured for 24 h. The medium was replaced with DME medium supplemented with 1% FCS, and test samples were added to each well. Following a 24-h culture, the concentration of HGF in the medium was measured using enzyme-immunoassay, as described elsewhere.[32]

## RESULTS

**Induction of *in vitro* invasion by HGF** Gallbladder cancer is highly lethal due to the rapid invasion and metastatic progression of the cancer cells. GB-d1 gallbladder carcinoma cell line was established using cells from a patient with gallbladder cancer. We have previ-

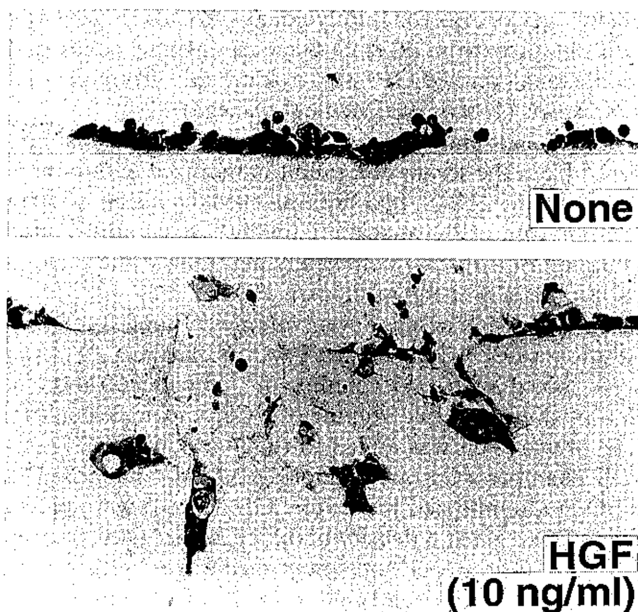


Fig. 1. Induction of the invasion of GB-d1 gallbladder carcinoma cells into collagen gel matrix in the presence of HGF. GB-d1 cells were plated on collagen gels at a density of $10^5$ cells/cm$^2$ and the cells were cultured for 12 days in the presence or absence of HGF. The medium was changed every 3 days. Cells were fixed, sectioned, and stained with hematoxylin.

ously shown that HGF stimulates the growth of GB-d1 cells, induces disruption of cell-cell junctions and stimulates the motility of cells, which results in scattering of the cells.[29] Thus, HGF has mitogenic and motogenic activities for GB-d1 cells.

The motogenic activity of HGF meant that HGF might affect the invasiveness of GB-d1 cells and we tested this possibility using an *in vitro* invasion model (Fig. 1). GB-d1 cells were cultured on top of a collagen gel matrix, arranged so that HGF penetrated the underlying collagen gel and reached the cells. Even though GB-d1 cell line was originally established from lymph node metastases of a gallbladder carcinoma and the GB-d1 cells invaded surrounding tissues when intradermally implanted into athymic mice (not shown), the cells did not invade the collagen gel matrix. However, GB-d1 cells invaded the collagen gels when cultured in the presence of 10 ng/ml HGF. Collagen gels were locally degraded along with invading cells, indicating that HGF may induce (and/or activate) proteases responsible for enzymatic degradation of collagen gels.

Since several growth factors are known to influence cell motility in other types of cells, we next examined whether the invasiveness of GB-d1 cells was affected by other typical growth factors, using the same culture method (Fig. 2). Although HGF strongly induced invasion of the cells, such a marked effect was not seen with the other growth factors used. EGF induced slight invasion, but its potency was far less than that of HGF, and TGF-β1, PDGF and bFGF did not induce invasion of cells. These results indicate that HGF is a potent invasion-promoting factor for GB-d1 cells and that this invasiveness is fairly specific to HGF, at least among the growth factors we used here.

**Enhancement of HGF production by GB-d1 cell-derived factor** During the course of the present study, we found that HGF production in normal human dermal fibroblasts was strongly stimulated by a soluble factor derived from GB-d1 gallbladder carcinoma cells. The conditioned medium of GB-d1 cells was added to cultures of normal skin fibroblasts and the HGF concentration in the culture medium was determined after 24-h culture (Fig. 3). Addition of the GB-d1 cell-derived conditioned medium dose-dependently stimulated HGF production by normal fibroblasts, thereby indicating that GB-d1 cells secrete soluble factor(s) which stimulate HGF production. We previously noted that IL-1 is a potent inducer of gene expression of HGF in dermal fibroblasts,[33] and, as expected, IL-1α strongly enhanced HGF production. The potential of the GB-d1 cell-derived factor to stimulate HGF production was similar to that of IL-1α.

Among various growth factors and cytokines, IL-1α, IL-1β, EGF, transforming growth factor-α (TGF-α), bFGF, and PDGF stimulated HGF production in the human skin fibroblasts used in the present study (not shown). We then asked whether the GB-d1 cell-derived soluble inducer(s) for HGF is a growth factor or a cytokine, using specific antibodies for the ligand or receptor. Among the antibodies tested, the stimulatory activity for HGF production in the conditioned medium was almost completely abrogated by anti-IL-1β antibody, but other antibodies against IL-1α, bFGF, PDGF and EGF receptor had no significant effect on the stimulatory activity in the conditioned medium of GB-d1 cells (Fig. 4). Thus, the soluble inducer derived from GB-d1 cells was concluded to be IL-1β.

**Mutual interaction and GB-d1 cell invasion** Based on our results, we designed an *in vitro* model for tumor-stromal interactions, as shown in Fig. 5. Dermal fibroblasts were cultured on collagen membrane, collagen gels were overlayed, and GB-d1 cells were cultured on top of the gel. In this model, direct cell-cell interactions were prevented, as the collagen gel was approximately 1 mm thick. When the cells were cultured for 12 days under this condition, GB-d1 cells invaded the collagen gel even in the absence of additional factors, except for preimmune IgG (Fig. 6). Notably, the invasion of GB-d1 cells was almost completely inhibited by anti-HGF antibody, while

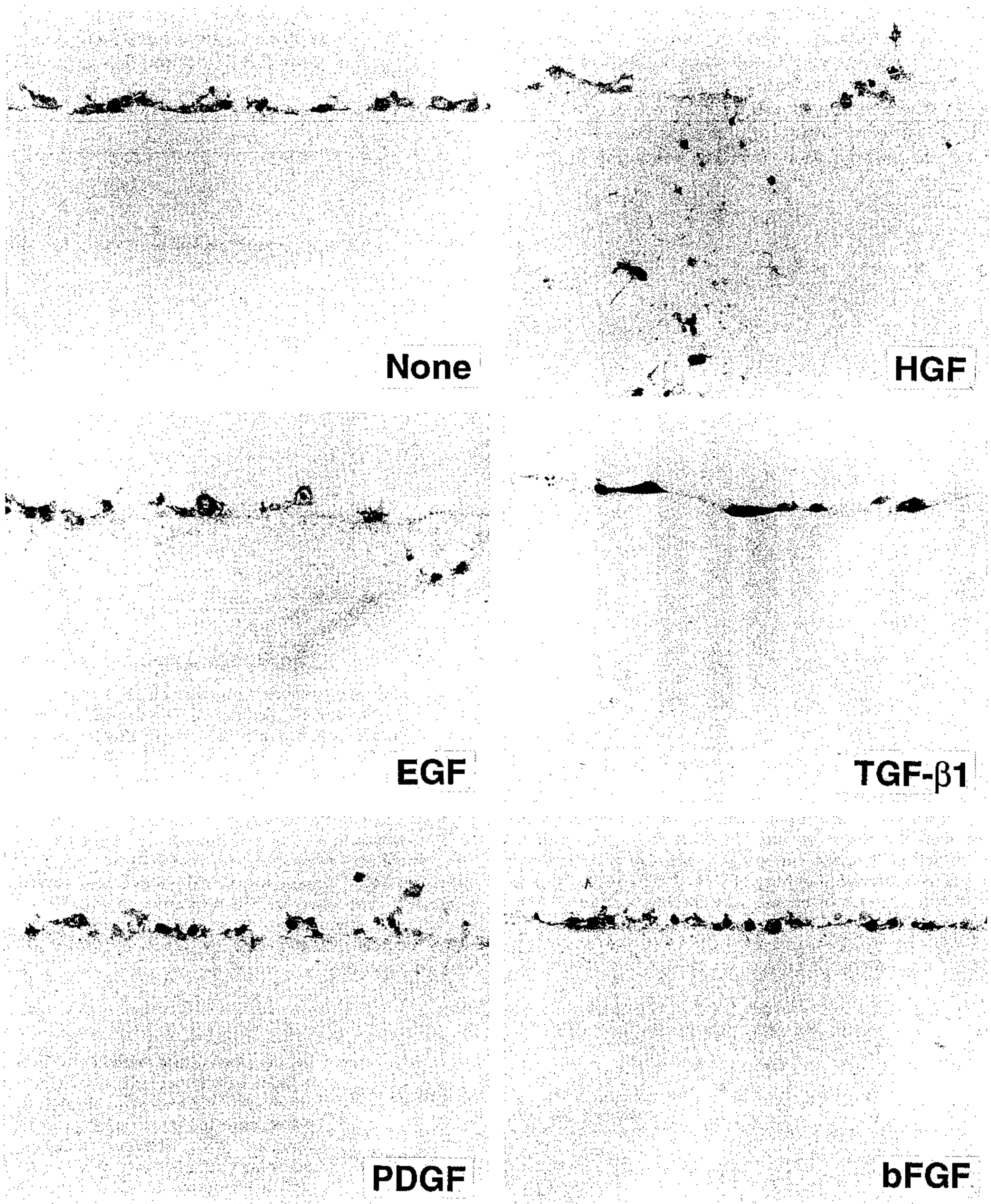


Fig. 2. Effect of growth factors on invasion of GB-d1 gallbladder carcinoma cells cultured on collagen gels. GB-d1 cells were plated on collagen gels at a density of $10^5$ cells/cm$^2$ and the cells were cultured for 12 days in the presence or absence of various growth factors. The medium was changed every 3 days. Growth factors were added at 10 ng/ml. Cells were fixed, sectioned, and stained with hematoxylin.

it was significantly inhibited by anti-IL-1β antibody. Although the stimulatory effect of conditioned medium from GB-d1 cells on HGF production in fibroblasts was almost completely abrogated by anti-IL-1β antibody (Fig. 4), the invasion of GB-d1 cells was inhibited by anti-IL-1β antibody to 50% of that in the control culture. Possibly the up-regulation of HGF production in fibroblasts by GB-d1-derived conditioned medium might be delayed in the co-culture system, since there is a significant lag period until GB-d1-derived IL-1β at effective

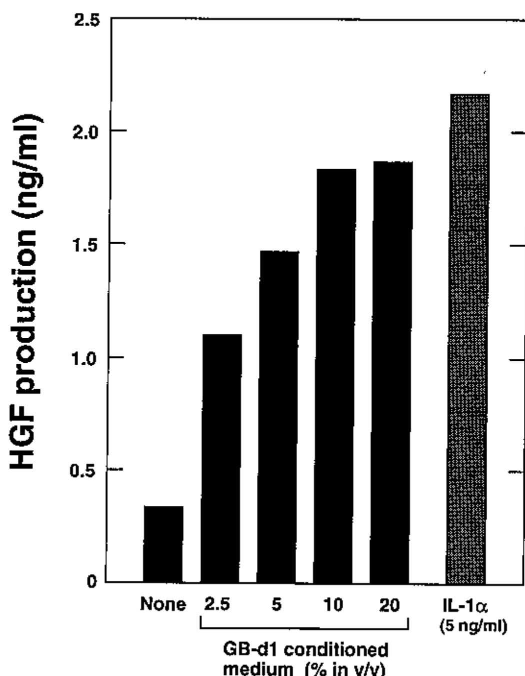


Fig. 3. Dose-dependent stimulation of HGF production in human skin fibroblasts by addition of conditioned medium from GB-d1 gallbladder carcinoma cells. Conditioned medium obtained after 48-h culture of GB-d1 cells at confluency was added to cultures of human skin fibroblasts and the cells were cultured for 24 h. HGF concentration in the medium of skin fibroblasts was measured by ELISA. IL-1α (5 ng/ml) was added as an authentic inducer for HGF production.[33]

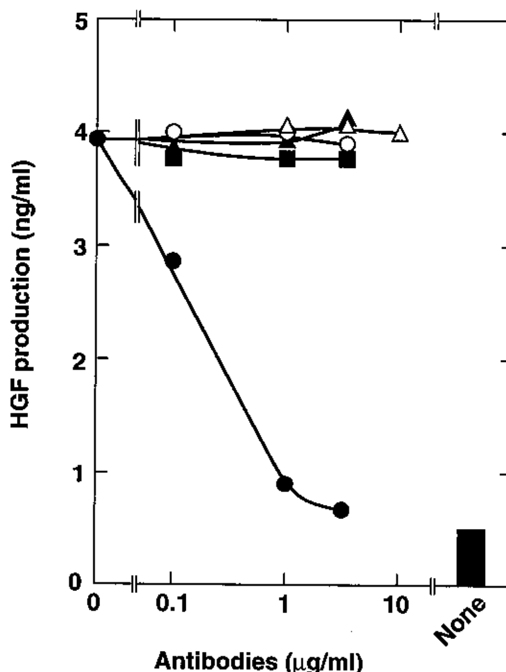


Fig. 4. Effects of various antibodies on HGF-inducing activity in the conditioned medium of GB-d1 gallbladder carcinoma cells. Conditioned medium from GB-d1 cells was preincubated with antibody against IL-1β (●), IL-1α (○), bFGF (▲), PDGF (△), or EGF receptor (■) for 30 min at 37°C and added to cultures of human skin fibroblasts at 10% (v/v). The fibroblasts were cultured for 24 h and the HGF concentration in the medium was measured by ELISA. The stimulatory effects of IL-1α, IL-1β, bFGF, and EGF at 1 ng/ml on HGF production in skin fibroblasts were almost completely suppressed by the respective antibodies at 1 μg/ml, and the stimulatory effect of PDGF at 1 ng/ml was almost completely suppressed by 3 μg/ml anti-PDGF antibody (not shown).

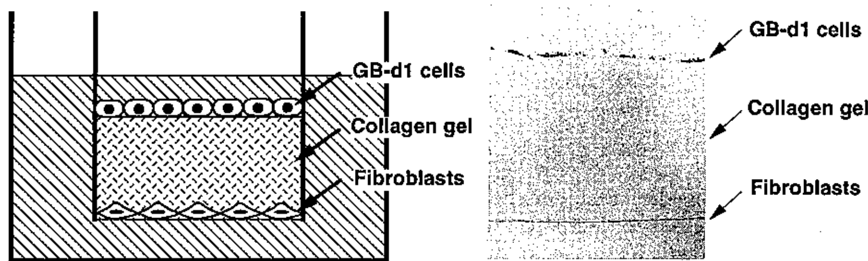


Fig. 5. Co-culture method of GB-d1 gallbladder carcinoma cells and human skin fibroblasts. Human skin fibroblasts were plated onto the inner cup of a Trans-well chamber with attached collagen film (Corning) at a density of $5\times10^4$ cells/$cm^2$ and cultured for 24 h. The collagen solution was overlayed and GB-d1 cells were plated on the collagen gel at a density of $10^5$ cells/$cm^2$. The right-hand photograph shows a transverse section of this co-culture system.

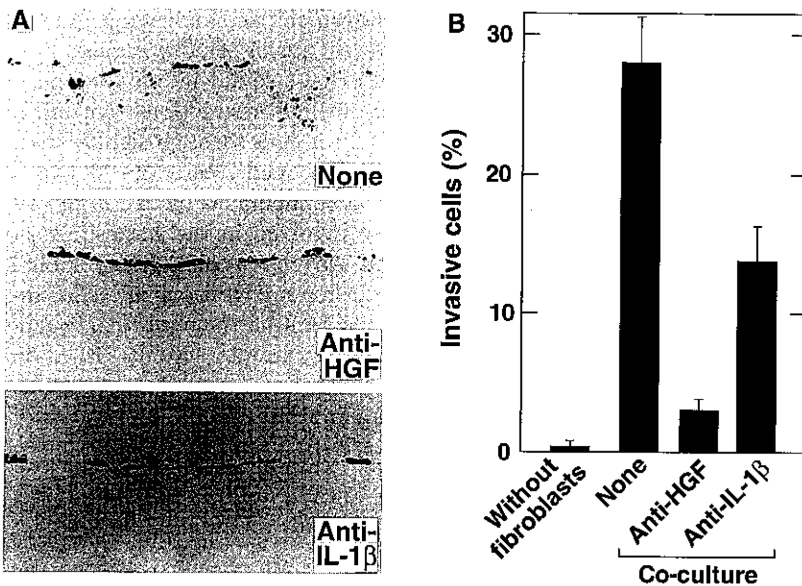


Fig. 6. Induction of GB-d1 cell invasion into collagen gels in the co-culture system with human skin fibroblasts and inhibition by antibodies against HGF and IL-1$\beta$. (A) Transverse sections of co-cultured GB-d1 cells in collagen gels. (B) The number of GB-d1 cells invading the gels in the co-culture system. GB-d1 gallbladder carcinoma cells were co-cultured with human skin fibroblasts as described in Fig. 5. These cells were cultured for 12 days in the presence of preimmune IgG, anti-human HGF IgG, or anti-human IL-1$\beta$ IgG (10 $\mu$g/ml each). The medium was changed every three days and the cultures were fixed, sectioned, and stained with hematoxylin. Photographs in A show typical fields in transverse sections. The number of cells invading the collagen gels was determined using 5 randomly selected sections, under each condition. The data represent the mean ($\pm$SD) of triplicate measurements.

concentrations affects co-cultured fibroblasts. The concentration of HGF in the upper medium of the inner cup was 2.9 ng/ml in the presence of preimmune IgG, and $<0.1$ ng/ml and 1.1 ng/ml in the presence of anti-HGF and anti-IL-1$\beta$ antibodies, respectively. Thus, the invasion of GB-d1 cells in the co-culture system seems to be consistent with the concentration of HGF in the medium. These results indicate that the fibroblast-derived invasion factor for GB-d1 cells is HGF, and HGF production in fibroblasts is up-regulated by IL-1$\beta$ derived from the cancer cells.

## DISCUSSION

Growth, invasion and metastatic potential of tumor cells are greatly influenced by tumor-stromal interactions, and the molecular and cellular mechanisms of these interactions are extremely complex. The stimulation of growth and invasion of carcinoma cells through interactions with stromal cells has been noted *in vivo* as well as *in vitro*. Co-cultivation of carcinoma cells on collagen gels in which fibroblasts are embedded induces invasion of tumor cells,[8] and coinoculation of carcinoma cells with fibroblasts into athymic mice results in an augmented proliferative and invasive potential of tumor cells.[6,7] These results can be interpreted as indicating that interactions between carcinoma cells and stromal fibroblasts are important in regulating growth and invasion of tumor cells. We recently identified a fibroblast-derived invasion factor functioning in oral squamous cell carcinoma cells as HGF,[34] and an earlier report claimed that HGF induces invasion of epithelial cells into collagen gels.[35] In the present study, we obtained evidence that HGF is the predominant fibroblast-derived factor which induces mitogenesis, motogenesis, and invasion of GB-d1 gallbladder carcinoma cells. Moreover, the carcinoma cells secrete an inducer of HGF production in fibroblasts. This inducer was identified as IL-1$\beta$.

Motogenesis (or migration) of cells and degradation of the extracellular matrix are key components for tumor invasion. Several studies suggested possible mechanisms for the motogenic action of HGF. HGF induces tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$),[34] by which cell spreading, focal adhesion formation and cell-matrix interactions are regulated. Activation of small GTP-binding proteins is essential for HGF-induced cell movement and cytoskeletal rearrangement.[36,37] HGF-induced tyrosine phosphorylation of $\beta$-catenin may regulate cadherin-dependent cell-cell interactions.[38,39] On the other hand, up-regulation of urokinase-type plasminogen activator and collagenase seems to be involved in degradation of components of the extracellular matrix.[40,41]

The establishment of an autocrine loop of growth factors and their receptors is known frequently to result in tumorigenic transformation of cells, and gene transfer experiments indicated that autonomous activation of Met/HGF receptor results in tumorigenic transformation.[42,43] Such an autocrine activation of Met/HGF receptor is involved in malignant transformation in certain tumor cells,[44,45] but most species of carcinoma cells do not secrete HGF (our unpublished result). Nevertheless, all our studies indicate that, irrespective of the autocrine activation of Met/HGF receptor, a paracrine interaction of HGF and Met/HGF receptor is important for the

malignant behavior of carcinoma cells. The overexpression or amplification of c-Met/HGF receptor gene was noted[46–48] and HGF regulates growth and motility in various types of carcinoma cells, presumably in a paracrine manner.[49–51]

A key finding in our study is that GB-d1 gallbladder cancer cells secrete an inducing factor for HGF production in stromal fibroblasts. This finding led to the notion that a looped interaction of tumor cells and stromal fibroblasts may exist, in which a tumor-derived factor regulates stromal HGF production while stromal-derived HGF regulates growth and invasion of tumor cells. Importantly, we recently found that various types of carcinoma cells secrete HGF-inducers and these HGF-inducers include at least IL-1$\beta$, IL-1$\alpha$, bFGF, and PDGF (our unpublished results). Other workers have also noted the presence and characterization of tumor-derived factors which stimulate HGF production.[52, 53] In this context, it is worth noting that HGF level in tissue extracts of breast tumor is a strong predictor of recurrence and survival in human breast cancer.[54] Since breast carcinoma cells do not express HGF,[55] the finding suggests that stromal-derived HGF may affect malignant phenotypes of breast carcinoma cells in a paracrine manner.

HGF is currently considered to be a crucial mediator in epithelial-mesenchymal (or -stromal) interactions, affecting cell growth, movement and morphogenesis of specific tissue architectures during embryogenesis and organ regeneration. Epithelial-stromal interactions may be functioning in tumor-stromal interactions that affect growth and invasion of tumor cells through signals from surrounding stromal cells. Looped interaction between tumor cells and stromal cells mediated by HGF and its inducers may possibly be involved in the acquisition of malignant phenotypes in various species of carcinoma cells. Identification and characterization of HGF-inducers are expected to shed light on the molecular mechanisms involved in tumor-stromal interactions. Based on our current results, the generation of antagonistic molecules may be a promising approach to the prevention of tumor invasion and metastasis.

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