Induction of Invasive Growth in a Gallbladder Cancer Cell Line by Hepatocyte Growth Factor *in vitro*

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To study the mechanism of invasion and metastasis of gallbladder cancer cells, we established a cancer cell line, GB-d1, from a metastatic lymphnode of poorly differentiated adenocarcinoma of the gallbladder. GB-d1 cells proliferate well in a dish culture and form small cystic cell clusters in a collagen gel containing 10% fetal bovine serum. A conditioned medium of human embryonic lung fibroblasts (HEL) stimulated the proliferation of GB-d1 cells and induced cell scattering in the dish culture. In the gel culture, the conditioned medium induced a transformation of the spherical clusters to arborizating colonies with tubular projections that mimicked an invasion of cancer cells into the surrounding tissue. Similar results were obtained when 10 ng/ml of human recombinant hepatocyte growth factor (h-rHGF) was added to the culture medium. The proliferative and morphological changes induced by the conditioned medium were inhibited by antiserum against h-rHGF. HEL and human gallbladder stromal fibroblast-like cells produced substantial levels of HGF in the culture media, while GB-d1 did not produce any detectable level of HGF. These results suggest that HGF promotes the invasive growth of gallbladder cancer cells *in vitro*, and it was also suggested that stromal fibroblasts may play an important role in the invasive progression of gallbladder cancer in a paracrine fashion.

Key words: HGF — Stromal fibroblast — Collagen gel — Gallbladder cancer cell line — Invasion

Gallbladder cancer is one of the most lethal of all digestive tract cancers even if a radical operation is performed. Its survival rate is very low (the median survival rate is 0.5 years while the 5-year survival rate is 13–30%) after diagnosis.[1,2] One reason for the poor prognosis of this cancer seems to be the rapid invasive and metastatic progression of the cancer cells.[3] However, the mechanism which induces the highly invasive activity of the biliary cancer cells is still not understood, because knowledge of the biological properties of cancer cells still remains insufficient. To study the mechanism of invasion of gallbladder cancer cells, we established a gallbladder cancer cell line (GB-d1) from a patient suffering from gallbladder cancer with multiple lymphnode metastasis.[4] GB-d1 cells proliferate with a population doubling time of about 24 h in a dish culture, and then form spherical colonies in the collagen gel matrices. At the early passages of this gallbladder cancer, the tumor cells grew invasively into the collagen gel matrix. However, in the course of the establishment of this cell line, the GB-d1 cells lost some of their invasive characteristics, such as infiltration into the gel matrix. The loss of invasive growth seemed to coincide with the disappearance of contaminating fibroblasts.[4]

We herein present the theory that the invasive growth of GB-d1 cells in the collagen gel matrices was induced by hepatocyte growth factor (HGF) secreted by human embryonic fibroblasts. Moreover, stromal fibroblast-like cells derived from human gallbladders were observed to secrete substantial amounts of HGF into the culture media. These results suggest that one reason for the high invasiveness of the gallbladder cancer cells may be their high susceptibility to HGF secreted by the stromal cells, during cancer-mesenchymal interaction.

MATERIALS AND METHODS

Cell and cell culture GB-d1 is an established cell line of poorly differentiated adenocarcinoma of the gallbladder. A metastatic lymphnode obtained from a 58-year-old man during an exploratory laparotomy was rinsed well with calcium-magnesium-free Hanks' balanced salt solution containing 100 μg/ml kanamycin (Meiji Seika Co., Tokyo), and then was chopped with scissors into several cubes measuring 1–2 mm. Half of each tumor cube was placed on a culture dish (Primaria, Falcon, Becton Dickinson Labware, Oxnard, CA) in William's medium E (WE) (Flow, Irvine, Scotland) supplemented with 100 μg/ml kanamycin and 10% fetal bovine serum (FBS) (Flow, North Ryde, Australia) and cultivated in a

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humidified CO_2 incubator. The other half of each cube was embedded in a collagen gel matrix and cultured as described below. The cells continued to proliferate on the dish culture and a new cell line, GB-d1, was thereby established.

HEL,[5] MRC-5 (MRC-5-30)[6] and TIGs (TIG-1-20, TIG-2M-30, TIG-3-30, TIG-7-50)[7, 8] (kindly provided by the Japanese Cancer Research Resources Bank, Tokyo) are fibroblasts derived from lungs of human embryos. All cell lines were frozen and stored at $-80°C$ until use.

For the collagen gel matrix culture, 0.4 ml of ice-cold WE medium containing 0.2% type 1 collagen (Type 1a, Nitta Gelatin Co., Osaka) and 10% FBS was settled at $37°C$ for 10 min in a 16 mm culture dish (16 mm multidish 4, Nunc, Roskilde, Denmark) as a basal gel layer. GB-d1 cells were detached from the culture dishes with 0.25% trypsin-EDTA solution and were suspended in 0.4 ml of the ice-cold collagen solution. The cell suspension in collagen was overlaid on the basal gel layer and cultured at $37°C$ by covering with 0.4 ml of WE medium supplemented with 10% FBS.

The primary cultures of the human gallbladder fibroblast-like cells were performed as follows: gallbladders surgically resected for cholelithiasis with mild inflammation were rinsed well with cold WE medium, and their mucosas and serosas were mechanically removed. The stromal tissue was minced and cultured in a WE medium containing 10% FBS in the dish culture. After three to four successive subcultures to remove any contaminating epithelial cells, confluent monolayers of spindle-shaped fibroblasts were obtained. Informed consent to the establishment of these human cell cultures was obtained pre-operatively in all cases.

Conditioned medium of human fibroblasts Human gallbladder fibroblast-like cells and embryonic fibroblasts (HEL, TIG and MRC-5) stored in a refrigerator at $-80°C$ were cultured in 60 mm dishes for several days. When the cells reached subconfluence, the medium was replaced with fresh medium (5 ml/dish) containing 10% FBS. The conditioned medium was collected after 48 h incubation, filtered through a Millipore filter (Minisart N 17597K, 0.2 μm, Sartorius, Göttingen, Germany), and stored at $-20°C$ until use.

Growth factors and antibody Recombinant human HGF (h-rHGF) was purified from culture fluid from Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA as described elsewhere.[9, 10] Lyophilized h-rHGF was dissolved in WE medium to a concentration of 1 μg/ml and used. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor (TGF)-β were purchased from Collaborative Biomedical Products (Bedford, MA), dissolved in WE medium and stored at $-20°C$ in small aliquots until use. Insulin was purchased from Novo Nordisk (Denmark). Polyclonal antibody against HGF was prepared from rabbits immunized by h-rHGF.[11] For neutralization experiments, the samples were incubated for 1 h at $25°C$ with the antibody diluted by 1:1000.

Cell proliferation assay Cells proliferating in the logarithmic phase were suspended in trypsin-EDTA, and 8,000–50,000 cells/dish were reseeded into 35 mm dishes either in duplicate or in triplicate. At 24 h after seeding, varying doses of growth factors, EGF, h-rHGF, TGF-β, bFGF or insulin, were added to each culture (at 0 h). After a further 72–96 h incubation, the cell number was determined by a Coulter counter (Coulter Electronics, Hialeah, FL).

Enzyme-linked immunosorbent assay (ELISA) The HGF concentrations in the conditioned media of GB-d1 or human fibroblasts were determined by ELISA.[12] Briefly, subconfluent cultures of these cells (0.75–1.30 × 10^6 cells/60-mm dish) were incubated in fresh medium at 5 ml/dish for 48 h and the culture media were collected. These conditioned media were serially diluted and incubated in multiwells pre-coated with rabbit anti-h-rHGF polyclonal antibody for 2 h at $37°C$. The wells were washed three times with PBS-Tween (phosphate-buffered saline containing 0.025% Tween 20), biotinylated polyclonal anti-human HGF IgG was added, and incubation was continued for 2 h at $37°C$. The reacting anti-human HGF IgG was measured by the horseradish peroxidase-conjugated streptoavidin-biotin complex-method.

Histological Examination Paraffin-embedded sections were prepared from either the original tumor, the cancer cubes cultured in collagen gel or the tumors that developed in nude mice, and then stained with hematoxylin and eosin (HE), or alcian blue at pH 2.5 and periodic acid-Schiff.[13]

RESULTS

Growth stimulation by the conditioned medium of human embryonic fibroblasts GB-d1 cells proliferated well on the plastic culture dishes with a doubling time of 23–25 h in the presence of 10% FBS. When 50% (v/v) of the conditioned media of human fibroblast lines (HEL, TIG-20, TIG-2M-30, TIG-3-30 and TIG-7-50) were added to the cultures, the GB-d1 cells proliferated even more rapidly. After 4 days of incubation in the presence of the conditioned media of these fibroblast cell lines, 1.6–1.9-fold higher cell densities were reached.

Effects of growth factors on GB-d1 proliferation Embryonic fibroblasts are known to secrete various cytokines which affect cell function, proliferation and morphology. We examined the effects of such growth factors as EGF,
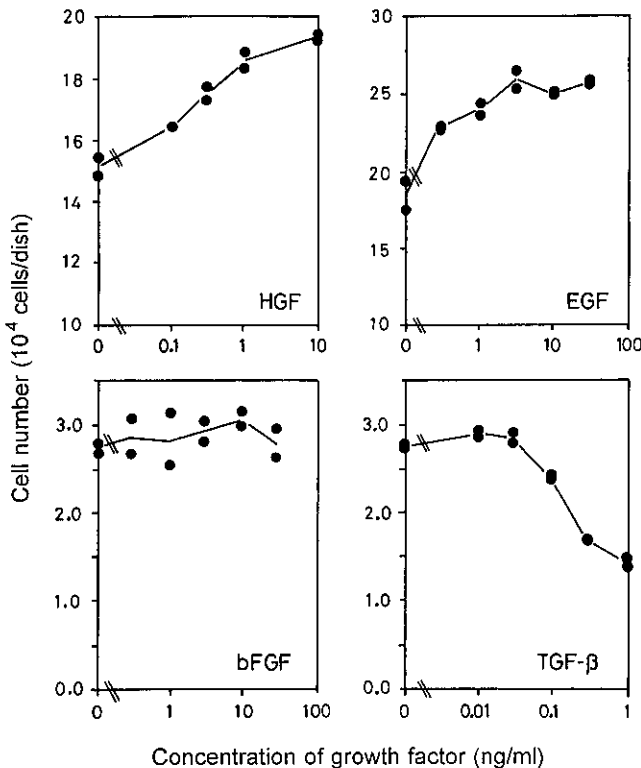
Fig. 1. The effect of various growth factors on the prolifera-
tion of GB-d1 cells. GB-d1 cells were cultured in the medium
containing various doses of EGF, HGF, TGF-β or bFGF for
either 96 h or 62 h. The cell number was determined by a
Coulter counter. In the cases of HGF and EGF, 20,000 cells/
dish were initially inoculated and incubated for 96 h. In the
cases of TGF-β and bFGF, 6,200 cells/dish were inoculated
and incubated for 62 h.

Table I. Growth Stimulation of GB-d1 Cells by HGF, EGF
and Insulin

Growth factors	Cell number after 96 h incubation (10³ cells/dish)	
	in 10% serum	in 3% serum
None	166.7±0.7 (1)[a]	136.9±3.1 (1)
HGF (10 ng/ml)	215.3±3.5 (1.29)	174.5±5.8 (1.27)
EGF (10 ng/ml)	221.1±1.9 (1.33)	170.3±1.8 (1.25)
Insulin (0.1 ng/ml)	165.0±7.9 (0.99)	136.6±2.7 (1.01)
HGF+EGF	218.2±4.5 (1.31)	157.2±2.1 (1.15)
HGF+EGF+insulin	211.8±1.7 (1.27)	159.2±2.0 (1.17)

The GB-d1 cells were replated at a cell density of 10,000
cells/35-mm dish and were cultured with medium containing
10% or 3% FBS. Growth factors were added to the cultures
singly or as a mixture and the cells were incubated for 96 h.
The cell number was then determined by a Coulter counter.
a) Relative cell number.

Table II. Growth Stimulation by HEL Fibroblasts and Its
Inhibition by Antibody against HGF

Culture medium	Cell number after 96 h incubation (10³ cells/dish)
50% HEL conditioned medium	182.0±6.6 (1.83)[a]
+antibody	142.9±5.7 (1.43)
10 ng/ml HGF	140.3±3.1 (1.41)
+antibody	95.5±1.8 (0.97)
Control (10% FBS)	99.6±0.3 (1)
+antibody	90.4±2.9 (0.91)

GB-d1 cells seeded to 35-mm dishes at a cell density of 8,400
cells/dish. The culture medium was replaced with the medium
indicated in the table and culture was continued for 96 h.
a) Relative cell number.

HGF, TGF-β, bFGF and insulin on the proliferation of
the GB-d1 cells. As shown in Fig. 1, EGF and HGF
dose-dependently stimulated the proliferation of GB-d1
cells. Maximal stimulation was obtained by 10 ng/ml of
HGF or 3–30 ng/ml of EGF. TGF-β inhibited the cell
proliferation at concentrations of 0.1–1 ng/ml. bFGF
did not affect the cell proliferation at concentrations of
0.3–30 ng/ml.

Additional effects of HGF, EGF and insulin on pro-
liferation were examined. The results in Table I showed
that either 10 ng/ml of HGF or 10 ng/ml of EGF
stimulated proliferation (about 1.3-fold increase), but no
additive effect was observed. Insulin (0.1 ng/ml) had no
stimulative or inhibitory effect. Even in a low serum (3%
FBS) condition, additive stimulation was not seen in the
mixtures of these factors, but rather a decrease in the cell
number was observed.

**Inhibitory effects of antiserum against h-rHGF on pro-
liferation and scatter of GB-d1 cells induced by condi-
tioned medium of HEL** The 50% conditioned medium
and HGF (10 ng/ml)-supplemented medium were both
preincubated with antiserum against h-rHGF for 1 h at
25°C. GB-d1 cells were cultured with these media and the
cell number was determined (Table II). After incubation
for 96 h, cells cultured in the conditioned medium
reached about 180×10³ cells/dish, and this cell density
was 1.8-fold higher than that in the control medium. The
pre-incubation of the conditioned medium with anti-
serum resulted in a partial reduction of the cell number.
The promotion of growth by 10 ng/ml of HGF was
completely neutralized by the antiserum to the control
level. The antiserum *per se* did not inhibit cell prolifera-
tion, as shown in the control experiment.

A phase contrast micrograph revealed that the GB-d1
cells changed their motility and became scattered in the
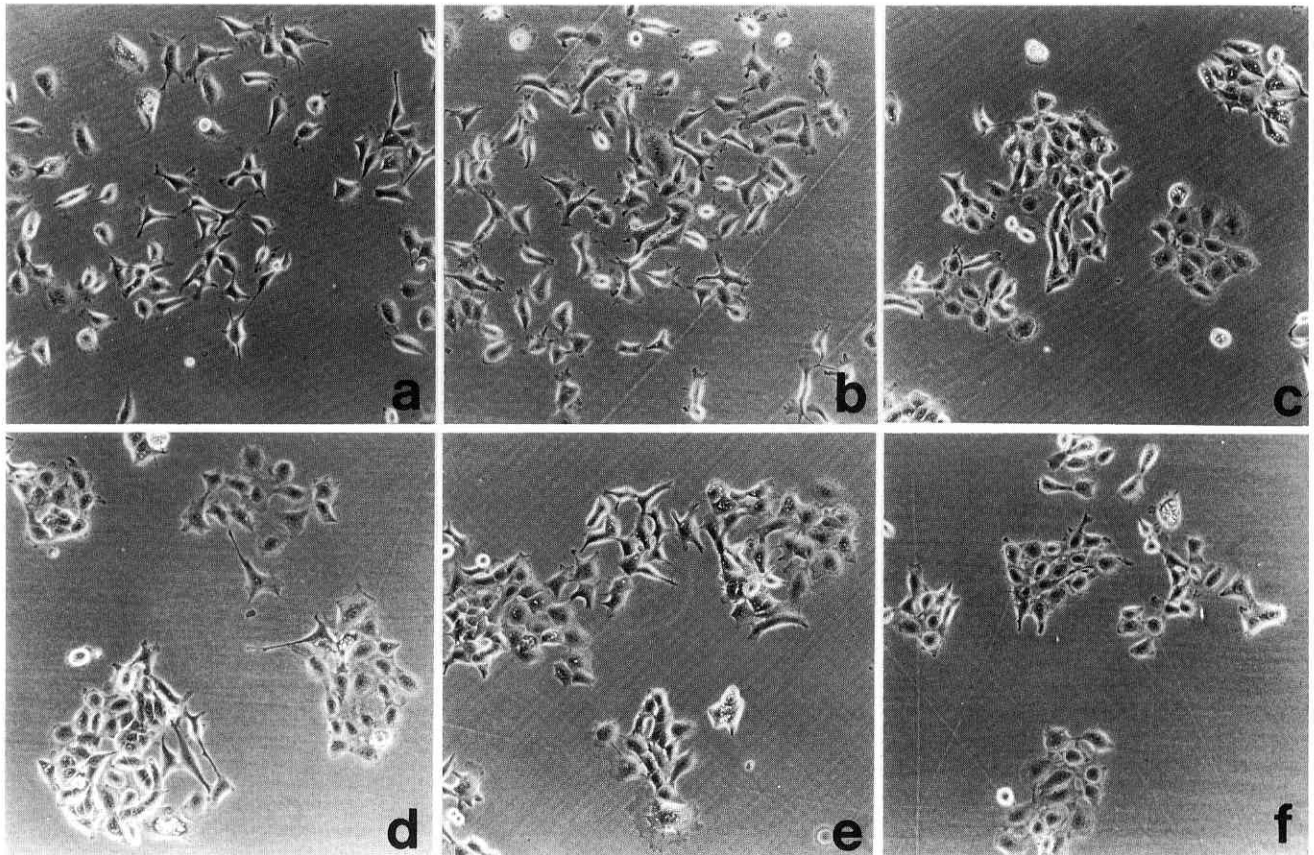dish culture during the 3-day incubation with the condi-

Fig. 2. The cell scatter induced by the conditioned medium or HGF, and its neutralization by the antibody against HGF. GB-d1 cells were incubated for 72 h with 10 ng/ml of HGF (a and d), with 50% (v/v) of the conditioned medium of HEL (b and e), or with only 10% FBS for the control (c and f). The culture media were preincubated with the antibody against h-rHGF (d, e and f).

tioned media of HEL (Fig. 2b) or TIG cells. Similar cell scattering was observed in the case of the addition of h-rHGF (Fig. 2a). Pre-incubation of the conditioned medium with the antiserum against h-rHGF completely inhibited the cell scattering (Fig. 2e).

Invasive growth of GB-d1 in the collagen gel matrix
When GB-d1 cells were embedded and cultured in type 1 collagen gel matrix at a low cell density (about 10^4 cells/ ml), the cells proliferated and formed spherical cell colonies (Fig. 3a). These cell colonies showed a cystic structure when thin sections of the colonies were examined by HE staining, and the cells of the cysts were round, while some of them were signet-ring type cells. In the presence of HGF 10 ng/ml, the shape of the colonies became quite different. The colonies of the GB-d1 cells were rapidly enlarged, and transformed from spherical to "coral-like" colonies with arborizing tubular structures (Fig. 3c). Incubation with the conditioned medium of

HEL caused the formation of similar coral-like colonies (Fig. 3b) while the coral-colony formation was completely inhibited by pre-incubation of the conditioned medium with anti-HGF serum (Fig. 3d).

Fig. 4 shows colonies that developed in the collagen gel matrix in the presence of various cytokines. Neither EGF (10 ng/ml), bFGF (10 ng/ml) nor TGF-β (0.1 ng/ml) was able to induce morphological changes such as the coral-like colonies observed in the case of HGF.

HGF production by human fibroblasts and GB-d1 cells
We determined the amount of HGF secreted in the culture medium of primary cultured fibroblast-like cells derived from human gallbladders and of embryonic fibroblasts (HEL and MRC-5) by ELISA as described in "Materials and Methods." The conditioned media of HEL and MRC-5 contained about 28 and 55 ng/ml of HGF, respectively. The conditioned media of the five human gallbladder stromal fibroblast-like cell cultures
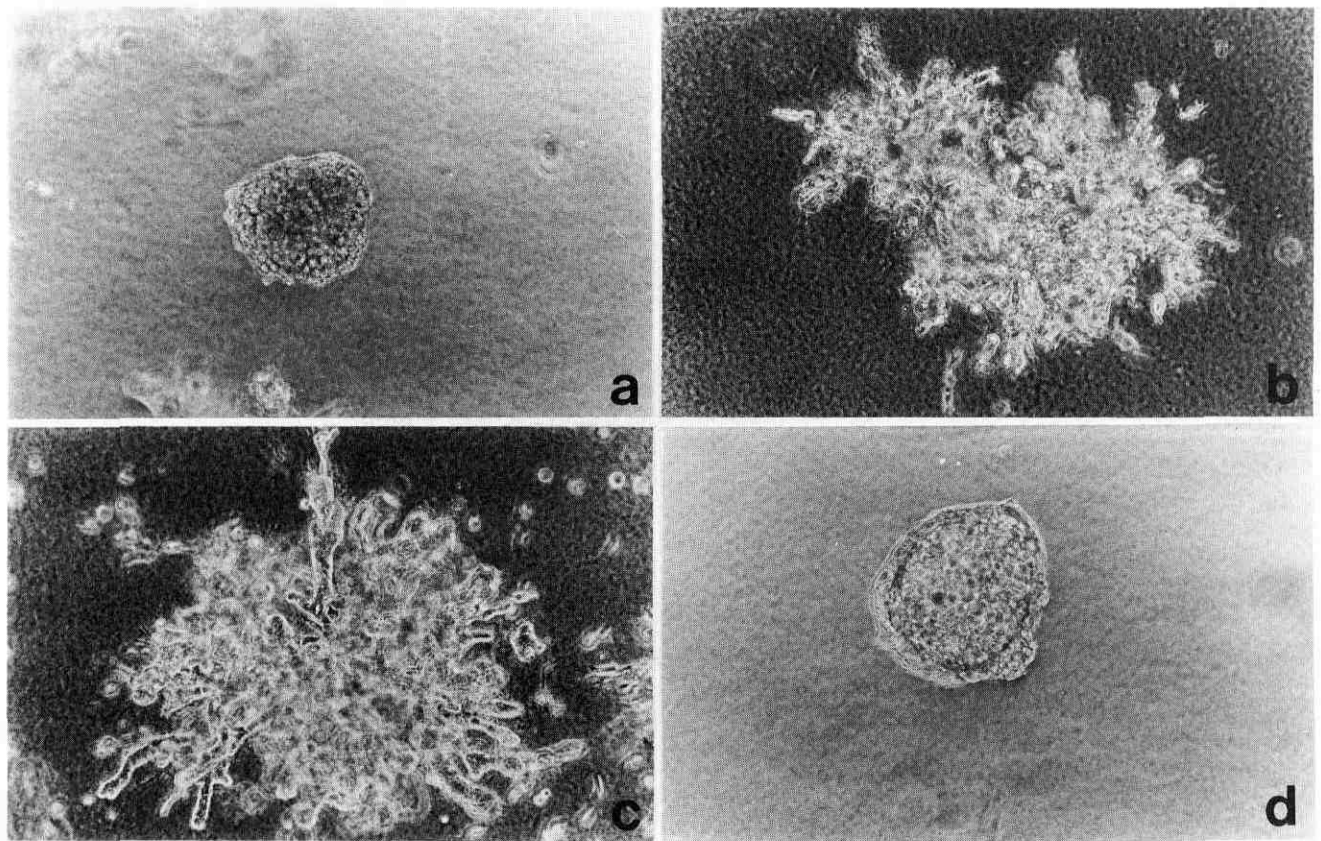
Fig. 3.   The colonies induced by the conditioned medium of embryonic fibroblasts in the collagen gel. GB-d1 cells were cultivated for 14 days in a type 1 collagen gel matrix (3,000 cells/0.4 ml gel) containing 50% conditioned medium of human embryonic fibroblasts (b and d), and either 0 (a) or 10 ng/ml (c) of HGF for the control. The conditioned medium was preincubated with anti-HGF antibody (d).

also contained about 6.9 ng/ml (2.4–13.5 ng/ml) of HGF. In contrast, GB-d1 cells did not produce any detectable level of HGF (less than 0.3 ng/ml).

Primary culture in the collagen gel matrix of a tumor developed by the transplantation of GB-d1 cells   A GB-d1 cell suspension injected subcutaneously into nude mice (5×10^6 cells/mouse) developed tumors within 2 weeks. These tumors morphologically appeared as poorly differentiated adenocarcinoma, similar to the original tumor in the patient. We again cultured the developing tumors in the collagen gel matrices by a similar procedure to that used for the primary culture of the original tumors of the patient. The tumor cubes measuring 1 mm in the gel matrices proliferated invasively with arborization into the gel, with the formation of tubular projections (Fig. 5b). This form resembled the tubular structure of the original tumor (Fig. 5a). In both primary gel cultures, contamination with many spindle-shaped fibroblast-like stromal cells was observed.

DISCUSSION

The invasion and metastasis of cancer cells are critical factors which aggravate the prognosis in cancer patients. An invasion of cancer cells depends on the proliferative ability and motility in the surrounding tissue when no basement membrane is present. We found in this study that GB-d1 cells, an established human gallbladder cancer line, are stimulated by both HGF and the conditioned media of human embryonic fibroblasts. HGF (10 ng/ml) accelerated the proliferation of GB-d1 cells and enhanced the cell motility, as reflected by cell scattering in dish culture (Fig. 2) and by invasive growth in the collagen gel matrix (Fig. 3). EGF, which induces cell DNA synthesis in many types of cultured epithelial cells, also stimulated GB-d1 cell proliferation in dish cultures. However, EGF does not induce invasive growth in cells from a collagen gel matrix culture. Basic FGF, TGF-β and insulin failed to accelerate cell growth either in a dish
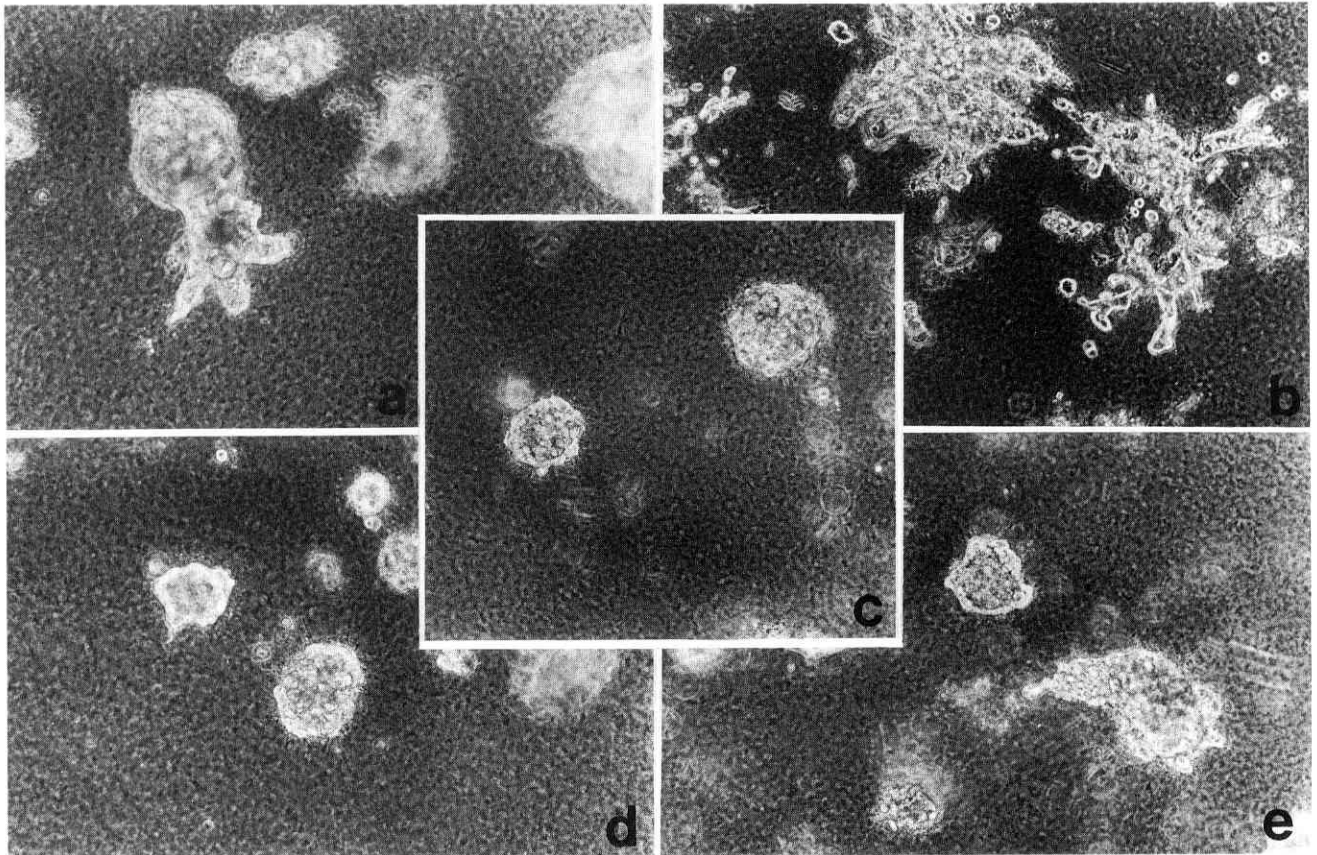
Fig. 4. Colony formation of GB-d1 cells in the collagen gel supplemented with various growth factors. GB-d1 cells were cultivated for 12 days in the type 1 collagen gel matrix (3,000 cells/0.4 ml gel) in the presence of 10 ng/ml of EGF (a), 10 ng/ml of HGF (b), 10 ng/ml of bFGF (d) or 0.1 ng/ml of TGF-β (e). Control (c).

culture or in a gel culture. Therefore, HGF was the strongest mitogen, motogen and morphogen for GB-d1 cells. To our knowledge, it has not been reported that biliary cancer cells are sensitive to HGF, and thus our gallbladder cell line, GB-d1, is the first cell line with this property.

HGF was purified by Nakamura and his colleagues as a factor which stimulates DNA synthesis in primary cultured mature hepatocytes.[14] Growth stimulation by HGF was also demonstrated in many types of cultured normal epithelial cells, such as human keratinocytes, and dog or monkey kidney epithelial cells (MDCK, BSC-1), in addition to mature hepatocytes, but not in non-epithelial fibroblasts.[15, 16] In contrast, growth stimulation of carcinoma cells by HGF is not so common. Although DNA synthesis of mature hepatocytes is induced by HGF, the growth of many hepatoma cell lines including HepG2 is inhibited.[17] Growth of some other cancer cell lines, IM-9 (human B-lymphoblasts), KB (human cervical carcinoma) and several colorectal cancer lines was

also suppressed by HGF.[18, 19] Thus HGF is believed to be a unique cytokine that suppresses cancer cell growth.[20]

We found that HGF induced drastic morphological changes in GB-d1 cells in the collagen gel matrix (Fig. 3). HGF, which is identical to scatter factor, stimulates the motility of epithelial cells: canine and monkey kidney cells (MDCK, BSC1), mouse breast cells and skin keratinocytes.[15, 21, 22] Montesano et al. reported that HGF caused non-transformed kidney epithelial cells (MDCK) to form arborizing tubular structures in a type 1 collagen gel culture.[11] They surmised that it resulted from tubular differentiation of MDCK cells induced by HGF. Johnson et al. reported that non-parenchymal epithelial liver cells proliferate and form colonies with arborization and tubularization in the collagen gel matrices in the presence of HGF.[23] In our case, thin sections of the GB-d1 colonies showed similar tubular structures in part (data not shown).

Although the reason for the susceptibility of GB-d1 cells to HGF is still not known, biliary tree epithelial cells
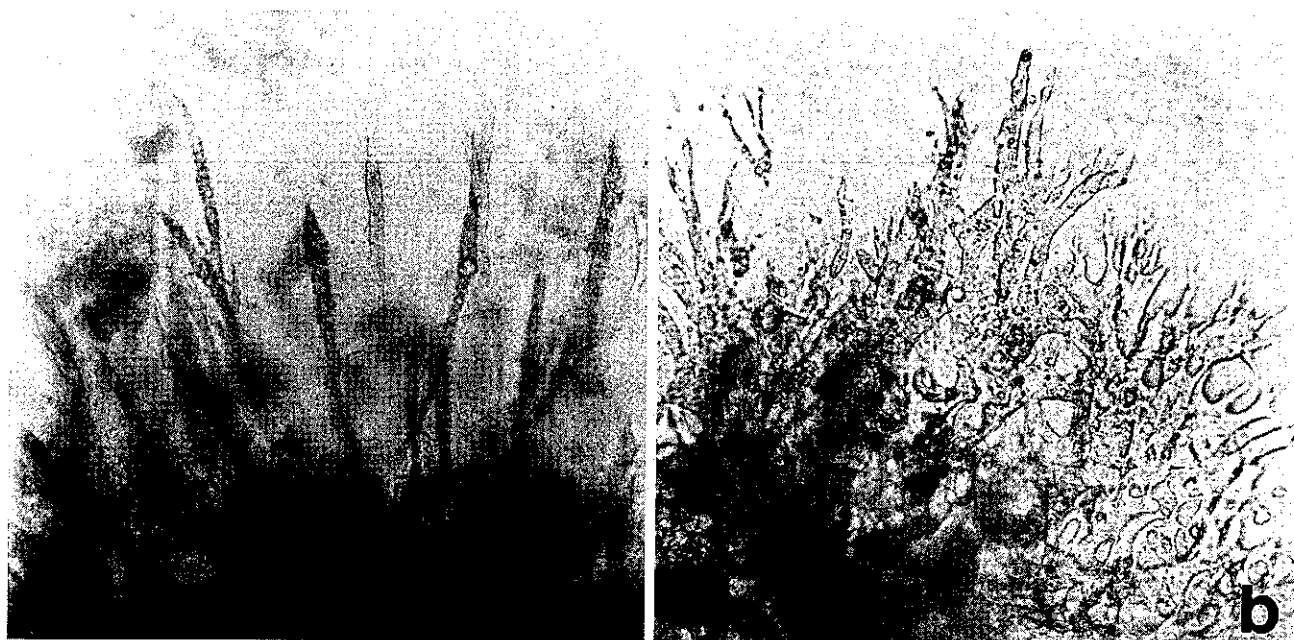
Fig. 5. Invasive growth of the gallbladder cancer tissue cultured in the collagen gel matrix. Gallbladder cancer tissue (a) (from which GB-d1 was established) and the tumor developed subcutaneously in a nude mouse after transplantation of GB-d1 cells (b) were chopped into small cubes measuring 1–2 mm and cultivated in collagen gel matrix for 5 days.

which include intra- and extra-hepatic bile ducts and gallbladder may be intrinsically sensitive to HGF. Joplin *et al.* reported that primary cultured bile duct epithelial cells proliferated in the presence of HGF.[24] Recently, we found that the DNA synthesis of rabbit primary gall-bladder epithelial cells was significantly stimulated by 10 ng/ml of h-rHGF (unpublished data). HGF may thus be a strong promoter of proliferation in both normal and cancerous biliary cells. This characteristic of biliary tree cells may be indispensable for coordinated liver regeneration.

Primary cultured fibroblast-like cells derived from human gallbladders produced HGF in the culture media. The HGF production by primary cultured gallbladder fibroblasts was as low as that by skin fibroblasts reported previously.[12] However, the concentration of 2 ng/ml is enough to stimulate GB-d1, as shown in Fig. 1. It is unlikely that the autocrine stimulation of cells by HGF

occurs in our case because GB-d1 did not produce a detectable level of HGF. It is therefore possible that the invasive growth of gallbladder cancer is induced by HGF which is secreted by the stromal cells in a paracrine fashion. In fact, the subcutaneous transplantation of GB-d1 cells into nude mice restored the invasive ability of cells into the collagen gel matrix (Fig. 5). In general, malignant neoplastic cells are characterized by invasive and metastatic progression. The control of the invasion associated with HGF may therefore result in an improvement of the prognosis of cancer patients.

ACKNOWLEDGMENTS

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