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Production of Basic Fibroblast Growth Factor-like Factor by Cultured Human Cholangiocellular Carcinoma Cells

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An extract of cultured human cholangiocellular carcinoma cells (HuCC-T1) was found to contain high mitogenic activity for BALB/c3T3 cells. The growth factor eliciting most of the mitogenic activity was purified and concluded to be identical with basic fibroblast growth factor (bFGF)-like factor on the basis of its molecular weight and heparin-Sepharose elution profile, and the results of immunoblotting and radioimmunoassay. HuCC-T1 cells also secreted bFGF-like factor into serum-free medium. A combination of insulin and transferrin or bovine serum albumin stimulated the growth of HuCC-T1 cells in serum-free medium. However, bFGF did not stimulate their growth in the presence and absence of these supplements. Neutralizing monoclonal antibody against bFGF did not inhibit growth. These results indicate that bFGF-like factor is not a growth factor for this cell line.

Key words: Basic fibroblast growth factor — Human cholangiocellular carcinoma

The development of new capillaries is important in the growth of solid tumors.[1] One growth factor stimulating tumor angiogenesis is thought to be FGF,[4] which is produced by tumor cells.[2,3] The FGF family are potent mitogens for a wide variety of mesoderm- and neuroectoderm-derived cells including endothelial cells and have been isolated from a variety of tissue and cell sources including tumor cells.[4,5] These factors can be grouped into two closely related classes, bFGF and aFGF. FGF exerts its biological response *in vitro* through high-affinity cell surface receptors.[5]

Transformed cells in culture acquire the ability to proliferate with little or no exogenous growth factor(s).[6] This phenomenon has been explained by the "autocrine hypothesis," which proposes that growth factors produced by the transformed cells act on their own cells via external receptors.[7,8] In support of this hypothesis, there are some reports that transformed cells secrete growth factors stimulating their own growth[9–11] and that specific antibodies against these growth factors or growth factor receptors inhibit growth of transformed cells.[12–14]

A serum-free culture system is useful in studies on the mechanisms controlling cell growth and the effects of growth factors.[15] We established a human cholangiocellular carcinoma cell line (HuCC-T1) that can grow in serum-free medium.[16] The present paper reports that a bFGF-like factor is present in HuCC-T1 cells and is also secreted from the cells, but that this factor, or neutralizing monoclonal antibody against bFGF that blocks its biological activity, does not affect the growth of these cells. On the other hand, a combination of insulin and transferrin or bovine serum albumin (BSA) stimulated the growth of these cells in serum-free medium.

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[4] Abbreviations used: FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; MAb, monoclonal antibody; BCE cells, bovine capillary endothelial cells; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Cells and culture A human cholangiocellular carcinoma cell line (HuCC-T1) was established as described previously.[16] The stock culture was maintained in Coon's modified Ham's F12 medium (C-F12) supplemented with 15 m*M* HEPES (pH 7.3), 100 units/ml penicillin, 100 μg/ml streptomycin and 3% calf serum (CS). The BALB/c3T3-3K cell line[17] was used for assay of the activity of bFGF stimulating DNA synthesis. The cells were cultured in Dulbecco's modified Eagle's medium (DME) containing 10% CS and all the other supplements described above. Bovine capillary endothelial (BCE) cells were isolated from bovine brain cortex and maintained as described by Goetz *et al.*[18] with slight modifications. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1 ng/ml bFGF and all the other supplements described above in dishes that had been coated with type-IV collagen (Sigma, St. Louis, MO), and were used for growth experiments at passages 5–9. All these cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Assay of DNA synthesis The activity of bFGF to stimulate DNA synthesis of cultured BALB/c3T3-3K cells was assayed as described previously.[17, 19] One unit of activity was defined as the amount equivalent to 1 mg of CS proteins in stimulating the incorporation of [^{3}H]-thymidine into DNA.

Growth experiments BCE cells were plated at a density of 2×10^4 in 5 ml of serum-free C-F12 medium in 60-mm Falcon dishes that had been coated with type-IV collagen. bFGF or test samples were added at the time of inoculation and on day 3. After 5 days, the cells were harvested by trypsinization and counted in a Coulter counter. HuCC-T1 cells were plated at a density of 2×10^4 in 5 ml of serum-free C-F12 medium in 60-mm Falcon dishes that had been coated with poly-D-lysine. Test samples were added only at the time of inoculation of the cells. After 5 days, cells were harvested by trypsinization and counted in a Coulter counter. Values are given as averages for duplicate experiments.

Purifications of bFGF and aFGF bFGF and aFGF were purified from bovine brain by the method of Gospodarowicz *et al.*[20] involving ammonium sulfate precipitation, and chromatographies on carboxymethyl (CM)-Sephadex and heparin-Sepharose. aFGF was further purified by cation-exchange HPLC on a Protein Pak G-SP column (8.2×75 mm) (Nihon Waters, Tokyo). The fractions eluted from the column with 0.35 *M* NaCl/50 m*M* sodium phosphate buffer (pH 6.8)/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS),[21, 22] which gave a single protein peak with DNA synthesis-stimulating activity, were collected. Protein concentrations of FGFs were determined with a Bicinchoninic Acid (BCA) kit (Pierce, Rockford, IL) with BSA as a standard.

Analysis of DNA synthesis-stimulating activities in cell extract (HuCC-T1-CE) and conditioned medium (HuCC-T1-CM) of HuCC-T1 cells For analysis of the mitogenic activities in HuCC-T1-CE and HuCC-T1-CM, their elution profiles on heparin affinity chromatography were examined.[21, 23] Confluent HuCC-T1 cells in 150-mm dishes were washed with protein-free C-F12 medium for 1 day and maintained in 20 ml of the medium for 4 days, and then the conditioned medium and cells were collected. The viability of the cells was more than 99%, as assessed by the trypan blue exclusion test. A crude extract of 10^7 HuCC-T1 cells in 2 ml of 10 m*M* Tris-HCl buffer (pH 7.5) and 15 ml of conditioned medium were applied to TSK Heparin-5PW HPLC columns (7.5×75 mm), which had previously been equilibrated with 10 m*M* Tris-HCl (pH 7.5) containing 0.5 *M* NaCl and 0.1% CHAPS. The columns were washed with 20 ml of the buffer and developed with a linear gradient of 0.5–2.0 *M* NaCl in the same buffer for 1 h at a flow rate of 0.8 ml/min. Fractions of 1.6 ml were collected and aliquots were assayed for stimulation of DNA synthesis in BALB/c3T3 cells as described above.

Purification of bFGF-like factor from cell extract (HuCC-T1-CE) and conditioned medium (HuCC-T1-CM) of HuCC-T1 cells A bFGF-like factor from HuCC-T1-CE was prepared by the methods of Schweigerer *et al.*[3] and Klagsbrun *et al.*[24] with some modifications. Briefly, 10^9 HuCC-T1 cells were harvested from monolayer cultures by trypsinization. The cells were suspended in 100 ml of 2 *M* NaCl/10 m*M* Tris-HCl (pH 7.5) containing 1 μg/ml leupeptin, 400 μ*M* pepstatin (both from The Peptide Institute Inc., Osaka), 1 m*M* phenylmethylsulfonyl fluoride (Sigma), 1 m*M* N-ethylmaleimide and 5 m*M* EDTA (both from Wako, Osaka). The cells were homogenized in a Potter-Elvehjem homogenizer for 3 min. The homogenate was centrifuged at 27,000*g* for 20 min, and the supernatant was dialyzed overnight against water to lower the concentration of NaCl to below 0.15 *M*. The dialyzed HuCC-T1-CE was adjusted to pH 6.0 and applied to a CM-Sephadex C-50 column (5×5 cm) (Pharmacia, Uppsala) that had been equilibrated with 0.1 *M* sodium phosphate buffer (pH 6.0). Material was then eluted stepwise with 0.1 *M* sodium phosphate buffer containing 0.15 *M* and 0.65 *M* NaCl. The material eluted with 0.65 *M* NaCl was applied to a heparin-Sepharose column (1.4×5 cm) (Pharmacia) that had been equilibrated with 0.65 *M* NaCl/10 m*M* Tris-HCl (pH 7.5)/0.1% CHAPS. The column was washed with about 5 column volumes of the same solution, and adsorbed material was then eluted with 500 ml of a linear gradient of 0.65–2.0 *M* NaCl in 10 m*M* Tris-HCl (pH 7.5)/0.1% CHAPS at a flow rate of 30 ml/h. Fractions of 10 ml were collected and tested for DNA synthesis-stimulating activity. The active fractions were combined, dialyzed against water and lyophilized. The powder was dissolved in 0.1% BSA/PBS and used for assays of biological activities and for immunological analyses. All operations were performed at about 4°C.

A bFGF-like factor from HuCC-T1-CM was prepared by the method of Schweigerer *et al.*[3] with some modifications. Briefly, HuCC-T1 cells were cultured in 850-cm^2 Falcon roller bottles with 100 ml of C-F12 containing 3% CS. After the cells had reached confluency (10^8 cells/bottle), they were washed with 100 ml of protein-free C-F12 for 1 day, and then the medium was replaced by 100 ml of fresh protein-free medium every 3 days for 2 weeks. The conditioned medium was clarified by centrifugation at 27,000*g* for 20 min and the resulting supernatant was stored at −20°C until use. Two liters of the conditioned medium was thawed, adjusted to pH 4.5 and stirred for 1 h at 4°C. Then it was centrifuged at 27,000*g* for 20 min, and the supernatant was adjusted to pH 6.0 and dialyzed overnight against water. After centrifugation at 27,000*g* for 20 min, the conditioned

medium was mixed with 300 ml of a packed suspension of CM-Sephadex C-50 in 0.1 *M* sodium phosphate buffer (pH 6.0). The suspension was stirred for 24 h and then poured into a column. The applied material was eluted from CM-Sephadex and purified by heparin-Sepharose chromatography as described for bFGF from the cell extract.

SDS-PAGE SDS-PAGE was performed by the method of Laemmli[25] in 19.5% polyacrylamide slab gel and protein bands were located with silver stain.

Immunoblots Proteins were separated by electrophoresis on SDS-12.5% polyacrylamide gel and then transferred electrophoretically to a nitrocellulose sheet. The nitrocellulose sheet was incubated first in blocking buffer (5% BSA/PBS) and then for 30 min with MAb against bFGF (bFM-2; 10 μg/ml)[26] diluted with washing buffer (0.1% Tween 20/PBS). The sheet was washed 4 times for 4 min each time with washing buffer and incubated with 500 ng/ml of biotinylated rabbit anti-mouse IgG (Vector Laboratories, Inc. Burlingame, CA) in PBS for 30 min. It was then washed again with washing buffer and incubated with horseradish peroxidase-avidin conjugate according to the manufacturer's instructions (Vector Laboratories, Inc.). The sheet was then washed extensively with washing buffer and the peroxidase activity was located with diaminobenzidine tetrahydrochloride.

Radioimmunoassay (RIA) Purified bovine bFGF was labeled with ^{125}I by the chloramine-T method and purified by heparin-Sepharose affinity chromatography.[26] The reaction mixture for RIA (0.5 ml) in a tube (Eiken, Tokyo) consisted of 0.35 ml of 0.1 *M* sodium phosphate buffer (pH 7.4)/0.02% NaN_3, 0.05 ml of 4 ng/ml ^{125}I-bFGF (8,000–15,000 cpm) in 0.1% BSA/PBS/0.02% NaN_3, 0.05 ml of unlabeled sample at an appropriate concentration in 0.1% BSA/PBS/0.02% NaN_3, and 0.05 ml of MAb against bFGF (bFM-1; 0.12 μg/ml)[26] in 0.1% BSA/PBS/0.02% NaN_3. After incubation overnight at 4°C, 0.1 ml of 1% normal mouse serum in PBS/0.02% NaN_3 and 0.1 ml of 0.77 mg/ml goat anti-mouse immunoglobulins (Dako, Glostrup) were added, and the incubation was continued for 4 h at 4°C. After addition of 1 ml of 0.2% PEG 6,000 (Nakarai Chemicals, Kyoto) the radioactivity bound to the antibody was precipitated by centrifugation and counted in an Aloka auto-well gamma system (ARC-300).

Other materials MAbs against bFGF[26] (bFM-1 and bFM-2) and hEGF (HA)[27] were obtained as described previously. Media and sera were products of Flow Lab. Inc., North Ryde, CA. Na^{125}I was obtained from the Radiochemical Centre, Amersham. Poly-D-lysine, human transferrin, defatted BSA and bovine insulin were obtained from Sigma.

RESULTS

Isolation of bFGF-like factor from HuCC-T1-CE and -CM We examined the activities to stimulate DNA synthesis in BALB/c3T3 cells of extracts of various cultured human tumor cell lines established from tumors of digestive organs, including hepatocellular carcinoma, pancreatic cancer and cholangiocellular carcinoma. HuCC-T1 cells, originating from a cholangiocellular carcinoma, had 3 to 60 times higher activity than the other cell lines examined (unpublished result). We then attempted to identify the growth factor showing this activity in an extract of the HuCC-T1 cells and also in conditioned medium obtained by culture of the cells in protein-free medium. The conditioned medium collected after maintaining 10^7 cells for 4 days in a 150-mm dish showed the activity stimulating DNA synthesis in BALB/c3T3-3K cells, when 10 μl of the conditioned medium was added to the medium for the assay. Its activity corresponded to 80 units/ml or 1.6 ng of bovine bFGF/ml when calculated on the basis of specific activity of purified bovine bFGF (5×10^7 units/mg protein). To analyze these activities, we subjected the cell extract and the conditioned medium to heparin-5PW HPLC column chromatography. As summarized in Table I, the

Table I. Distribution of Activities to Stimulate DNA Synthesis in BALB/c3T3 Cells in an Extract and Conditioned Medium of HuCC-T1 Cells[a]

	Activity (unit)/10^7 cells	Distribution on heparin-HPLC (%)[b]			Yield (%)
		non-FGF[c]	aFGF[d]	bFGF[e]	
Conditioned medium	1,600/4 days	0	0	100	43
Cell extract	4,500	1	0	99	64

a) Experimental conditions were as described in the text.
b) The total activity of all fractions was taken as 100%.
c) Activity in unadsorbed fractions and fractions eluted with a concentration of NaCl of less than 0.5 *M*.
d) Activity in fractions eluted with 0.9–1.1 *M* NaCl.
e) Activity in fractions eluted with 1.3–1.5 *M* NaCl.

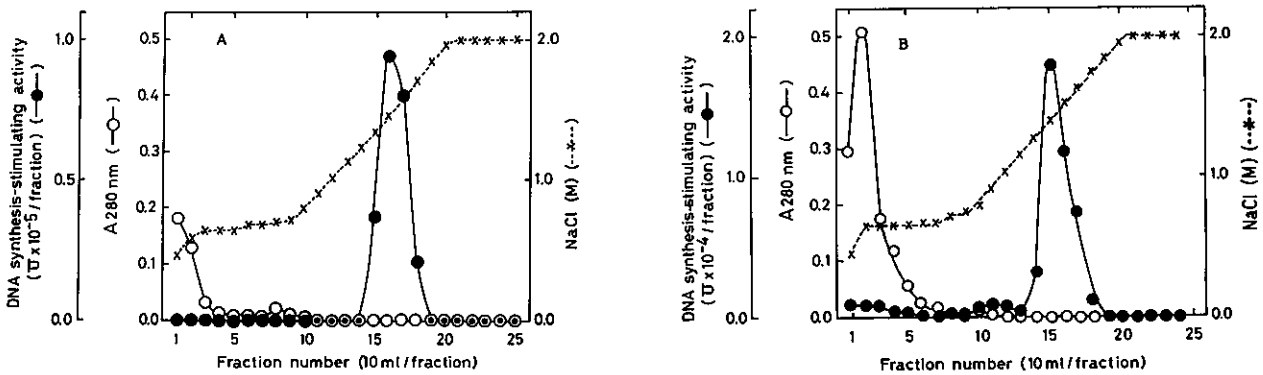

Fig. 1. Heparin-Sepharose affinity chromatography of the growth factor from an extract (A) and conditioned medium (B) of HuCC-T1 cells. Experimental conditions were as described in the text. The elution profile after washing the column with 0.65 *M* NaCl is shown.

activity in the cell extract was about 3 times that in the conditioned medium obtained by culture of confluent cells in protein-free medium for 4 days. Almost all the activity of both the extract and the conditioned medium bound to the heparin column and was eluted with 1.3–1.5 *M* NaCl, indicating that the activity was due to a bFGF-like factor.[23] The viability of the cells after collection of the medium was 99%, as assessed by the trypan blue exclusion test, and lysis and detachment of the cells were hardly observed during the culture period, indicating that the activity in the conditioned medium was not released from dead cells but secreted from live cells.

We purified these growth factors to confirm their identity with bFGF. When the cell extract was applied to a CM-Sephadex column, most of the activity was adsorbed on the column, and was eluted with 0.65 *M* NaCl (data not shown). The major active fractions were combined and applied to a heparin-Sepharose column (Fig. 1A). One peak of activity was eluted with 1.4–1.6 *M* NaCl. By these steps, 14 μg of bFGF-like factor was purified from 10^9 HuCC-T1 cells with about 30% recovery.

To purify the FGF-like factor in HuCC-T1-CM, we collected the conditioned medium from roller bottles on which HuCC-T1 cells were grown to confluency. The activity accumulated in the medium in a 4-day period varied from 60 to 80 units/ml. The activity was adsorbed on CM-Sephadex and eluted from the column with 0.65 *M* NaCl. The major active fractions were combined and applied to a heparin-Sepharose column, and one peak of the activity, like that from HuCC-T1-CE, was eluted with 1.4–1.6 *M* NaCl (Fig. 1B). By these steps, 1.5 μg of bFGF-like factor was purified from 2 liters of conditioned medium with about 20% recovery.

Characterization of bFGF-like factors We obtained two MAbs[26] against bovine bFGF, designated as bFM-1 and bFM-2, which blocked its biological activity. These MAbs were highly specific for bFGF from bovine, human and mouse sources, and did not cross-react with bovine aFGF. Moreover, we showed that bFM-1 recognized the conformation of the bFGF molecule necessary for its biological activity, whereas bFM-2 recognized the denatured bFGF. The concentration of bFGF for half-maximal displacement in RIA with bFM-1 was about one-tenth of that with bFM-2. Therefore, we assayed the bFGF-like factors using bFM-1. Fig. 2A shows that the proteins from HuCC-T1-CE and -CM both competed with radiolabeled bovine bFGF for binding to bFM-1 in a dose-dependent manner and that the displacement curves with the two bFGF-like factors were almost identical to that with purified bovine bFGF, when the doses were expressed as activity units. These results indicate that the specific activities of these growth factors were almost the same. The specific activity of purified bovine bFGF was $2.5–5 \times 10^7$ unit/mg protein, and those of the two purified bFGF-like factors were estimated to be about the same by determination of the protein concentrations of the samples, although these determinations were not accurate because the concentrations of the proteins were low. For immunoblot analysis of the bFGF-like factors, bFM-2 was used as a probe, because bFM-1 did not recognize denatured bFGF transferred to a nitrocellulose sheet. The cross-reactivities of the proteins with bFM-2 were almost the same as that of bovine bFGF, but aFGF did not cross-react with the antibody (Fig. 2B). The bFGF-like factor from the cell extract migrated as a doublet with apparent *Mr*s of 19,000 and 20,000. The factor from the conditioned medium also

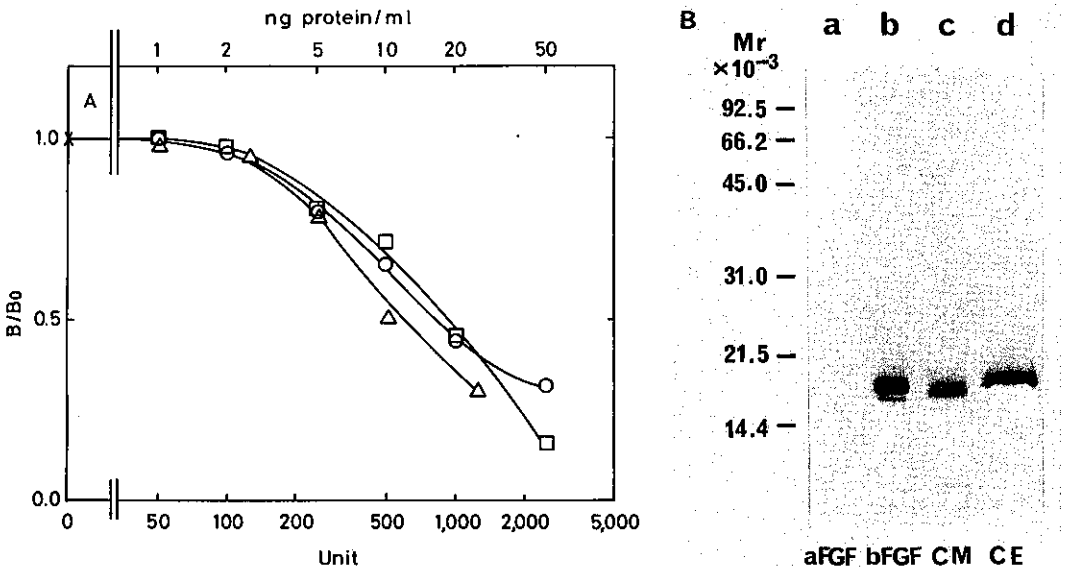

Fig. 2. (A) Cross-reactivities of bFGF (HuCC-T1-CE) and bFGF (HuCC-T1-CM) in RIA with MAb against bFGF (bFM-1). RIA was carried out as described in the text. Values of B (specific binding of ^{125}I-labeled bFGF in the presence of various concentrations of antigens) divided by B_0 (specific binding in the absence of unlabeled antigen) are plotted. The amount of bFM-1 added to the reaction mixture (0.5 ml) was 6 ng. The values of B_0 were 56% of the total tracer added. ○, bFGF (bovine); △, bFGF (HuCC-T1-CE); □, bFGF (HuCC-T1-CM). Units were determined by assay of DNA synthesis-stimulating activity as described in the text. (B) Immunoblot analyses of bFGF (HuCC-T1-CE) and bFGF (HuCC-T1-CM) using MAb against bFGF (bFM-2). Experimental conditions were as described in the text. Lane a, bovine aFGF (1 μg); lane b, bovine bFGF (1 μg); lane c, bFGF (HuCC-T1-CM) (0.5 μg); lane d, bFGF (HuCC-T1-CE) (0.5 μg). The following *Mr* markers from Bio-Rad (Richmond, CA) were used: phosphorylase B (*Mr*, 92,500), BSA (*Mr*, 66,200), ovalbumin (*Mr*, 45,000), carbonic anhydrase (*Mr*, 31,000), soybean trypsin inhibitor (*Mr*, 21,500), and lysozyme (*Mr*, 14,400).

gave two bands with apparent *Mr*s of 18,000 (major component) and 19,000 (minor component). We also examined the bFGF-like factors by means of SDS-PAGE and silver staining (Fig. 3). The proteins showed similar migration patterns to those seen on immuno-staining, although the sample of bFGF-like factor from conditioned medium gave some additional minor protein bands. bFGF was first reported to be a single-chain polypeptide composed of 146 amino acids,[28] but later some other forms that were truncated (131 or 135 amino acids)[24, 29] or extended (154 or 157 amino acids)[30, 31] at the amino terminus were also reported. It has been

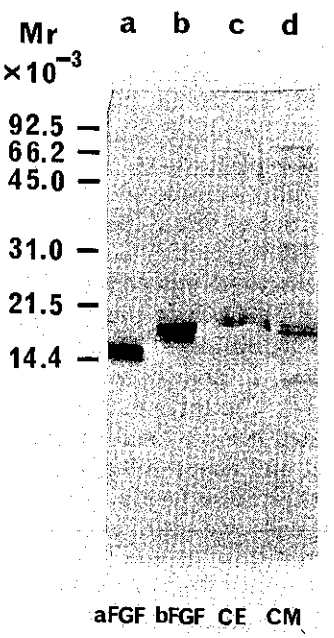

Fig. 3. SDS-PAGE of bFGF-like factors from an extract and conditioned medium of HuCC-T1 cells. Experimental conditions were as described in the text. Proteins were located with silver stain. Lane a, bovine aFGF (1 μg); lane b, bovine bFGF (1 μg); lane c, bFGF (HuCC-T1-CE) (0.5 μg); lane d, bFGF (HuCC-T1-CM) (0.5 μg). The *Mr* markers used were the same as for Fig. 2.

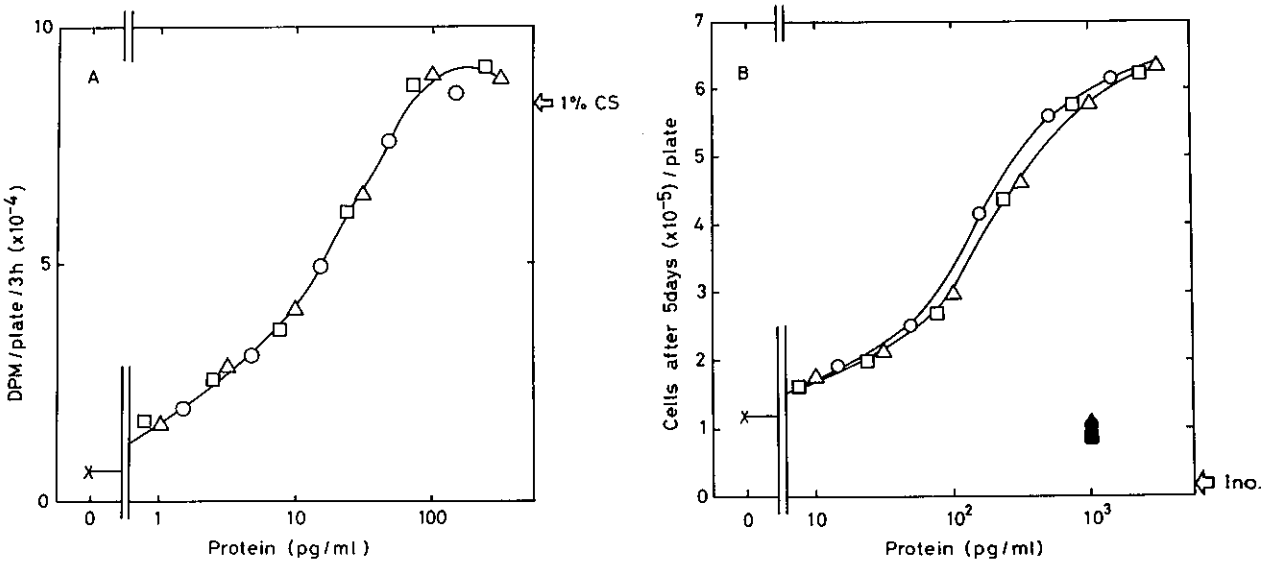

Fig. 4. Effects of bFGF-like factors from an extract and conditioned medium of HuCC-T1 cells in stimulating DNA synthesis in BALB/c3T3-3K cells (A) and growth of BCE cells (B). Experimental conditions were as described in the text. The protein concentration of bovine bFGF was determined with a BCA kit (Pierce, Illinois) with BSA as a standard, while those of bFGF (HuCC-T1-CE) and bFGF (HuCC-T1-CM) were estimated from the results of RIA. ○, bFGF (bovine); △, bFGF (HuCC-T1-CE); □, bFGF (HuCC-T1-CM); ●, bFGF+MAb (bFM-1) (10 μg/ml); ▲, bFGF (HuCC-T1-CE)+MAb (bFM-1) (10 μg/ml); ■, bFGF (HuCC-T1-CM)+MAb (bFM-1) (10 μg/ml); ×, No addition. The thick arrow in (A) indicates the activity in the presence of the optimal concentration (1%) of CS. The thick arrow in (B) (Ino.) indicates the number of cells inoculated.

suggested that the amino-terminal sequence of bFGF is apt to be cleaved by protease and that this sequence is not necessary for its biological activity. bFGF-like factor from the cell extract was purified in the presence of several protease inhibitors, but the factor from the conditioned medium was purified after being incubated in the medium probably containing proteolytic activity for a few days at 37°C. Therefore, the lower *Mr* of the factor from the conditioned medium than that from the cell extract may be due to proteolytic cleavage.

Biological activities of bFGF-like factors As described above, the bFGF-like factors purified from HuCC-T1-CE and -CM were potent mitogens for BALB/c3T3 cells. The dose-response curves of the activities to stimulate DNA synthesis in BALB/c3T3 cells of the bFGF-like factors are shown in comparison with that of bovine bFGF in Fig. 4A. The three curves were indistinguishable and indicated half-maximal and maximal effects at about 15 pg/ml and about 100 pg/ml, respectively. To determine whether these factors had similar mitogenic effects on BCE cells, we tested the effects of increasing concentrations of these factors and bovine bFGF on growth of BCE cells (Fig. 4B). Similar dose-response curves were again obtained and indicated half-maximal and maximal effects at about 200 pg/ml and 2 ng/ml, respectively. These results also support the conclusion that the bFGF-like factors from HuCC-T1-CE and -CM are indistinguishable from bFGF. MAb against bFGF (bFM-1) inhibited the growth of BCE cells stimulated by bFGF or bFGF-like factors from HuCC-T1-CE or -CM.

Effects of bFGF-like factors on growth of HuCC-T1 cells As reviewed by Barnes and Sato,[15] many kinds of cells can grow in serum-free medium supplemented with hormones, growth factors, and other defined materials. We studied the dose-dependences of the effects of some of these essential factors on growth of HuCC-T1 cells in serum-free C-F12 medium in poly-D-lysine-coated dishes. Poly-D-lysine-coated dishes were used extensively and growth was faster in these dishes than in uncoated dishes (data not shown), as in the case of A431 cells.[32] Table II summarizes results on the cell numbers after growth with optimal concentrations of these factors for 5 days. No growth stimulation was observed when bFGF or transferrin was added alone. Addition of insulin or BSA alone resulted in slight stimulation of cell growth. A combination of insulin and transferrin or BSA stimulated growth to nearly that in the presence of an optimal concentration (1%) of CS. No growth stimulation was

Table II. Effects of Various Additions on Growth of HuCC-T1 Cells in Serum-free C-F12 Medium[a]

Addition (μg/ml)	% of control cell number
None[b]	(100)
bFGF (0.003)	100
T (10)	100
I (10)	182
BSA (100)	155
T+I (10, 10)	556
T+BSA (10, 100)	185
I+BSA (10, 100)	686
I+BSA (1, 10)	441
I+BSA+bFGF (1, 10, 0.003)	433
T+I+BSA (10, 10, 100)	614
1% CS	581

a) Experimental conditions were as described in the text.
b) The cells were plated at a density of 2×10^4/dish. The cell number after 5 days in the control culture ($3.1–6.0\times10^4$ cells/dish) without any additions was taken as 100%. T=transferrin. I=insulin.

Table III. Effects of Various Additions on Growth of HuCC-T1 Cells in Serum-free C-F12 Medium Supplemented with Insulin and BSA[a]

Addition (μg/ml)	% of control cell number
None[b]	(100)
Bovine bFGF (0.003)	84
bFGF from HuCC-T1-CE (0.003)	108
bFGF from HuCC-T1-CM (0.003)	100
hEGF (0.1)	103
MAb (bFGF) (10)	161
MAb (hEGF) (10)	132

a) Experimental conditions were as described in the text.
b) The cells were plated at a density of 2×10^4/dish. The cell number after 5 days in the control culture ($2.1–3.6\times10^5$ cells/dish) with 10 μg/ml of insulin and 100 μg/ml of BSA in serum-free C-F12 medium was taken as 100%.

again observed when bFGF was added with sub-optimal concentrations of insulin (1 μg/ml) and BSA (10 μg/ml) which resulted in sub-optimal growth stimulation. Addition of higher concentrations of bFGF (up to 30 ng/ml) did not have any effect (data not shown). Transferrin and BSA with and without insulin did not have additive effects.

To determine whether the bFGF-like factors produced by HuCC-T1 cells act as an auto-stimulatory factor, we examined the effects of these factors and of MAb against bFGF (bFM-1) on growth of HuCC-T1 cells in the presence of insulin and BSA. Table III summarizes the cell numbers at the highest concentrations of these additives determined from dose-response curves. The bFGFs from bovine brain, HuCC-T1-CE and -CM did not stimulate growth, even at concentrations that caused maximal growth stimulation of BCE cells as described above. We looked for FGF receptor[5] in HuCC-T1 cells with ^{125}I-labeled bFGF and aFGF, but it could not be detected even after washing these cells with suramin or sodium acetate buffer (pH 4.0) containing 2 *M* NaCl. This result seems to be consistent with the lack of effect of bFGF on growth of these cells (Table II and Table III). hEGF also had no stimulatory effect. Moreover, although the MAb, bFM-1, completely inhibited growth of BCE cells at 10 μg/ml in the presence and absence of bFGF,[26] it did not inhibit, but slightly stimulated the growth of HuCC-T1 cells. MAb against hEGF at 10 μg/ml also slightly stimulated the growth of HuCC-T1 cells. The reason for the stimulatory effects of these IgG_1 preparations is unknown. These results indicate that the bFGF-like factor, at least the one that is secreted by HuCC-T1 cells, does not act as an autocrine growth factor.

DISCUSSION

In the present study, we showed that cultured human cholangiocellular carcinoma cells, HuCC-T1, produced a bFGF-like factor that was immunologically indistinguishable from bovine bFGF. We further found that this factor was not only associated with the cells, but also was secreted into the medium. There is no leader peptide region, which facilitates secretion, in the amino acid sequence of bFGF deduced from its cDNA nucleotide sequence.[33] Therefore, leaderless bFGF is probably produced inside the cells, carried to the cell surface with glycosaminoglycan as shown by several reports[34–36] and then released from the extracellular matrix by lysis with heparinase-like enzyme produced by the cells,[37] or by some unknown mechanism. Neither bFGF nor neutralizing MAb against bFGF affected the growth of HuCC-T1 cells in serum-free medium, suggesting that this factor in the conditioned medium does not act as autocrine growth factor. bFGF secreted from the tumor cells may function in inducing hyperplasia of adjacent normal tissue and angiogenesis and contribute to the development of solid tumors.

Recent studies have shown that three different oncogenes, int-2, hst/KS3 and FGF-5, encode FGF-homologous proteins.[38–41] These three proteins and bFGF share considerable amino acid sequence homol-

ogy, ranging from 35 to 55% identity in commonly aligned regions. However, these oncogene products have been found in only certain kinds of tumor cells. On the contrary, bFGF has been found in almost all cell lines derived from solid tumors examined.[42] The possibility of an autocrine role of bFGF in the growth of tumor cells has been suggested from studies on a human embryonal rhabdomyosarcoma cell line[3] and rat primary fibrosarcoma cells[43]: these cells produce bFGF that stimulates their own growth. In addition, transformation of cells without a requirement of bFGF for their growth has been achieved by introduction of bFGF cDNA into NIH 3T3 cells,[44] baby hamster kidney-derived (BHK) cells[45] and BALB/c 3T3 cells.[46] The conditioned media of these transformed cell lines producing bFGF was shown to contain a growth factor,[3,45,46] but the factor was not identified[46] or not quantitated.[3,45] Moreover, neutralizing polyclonal antibody against bFGF was found to inhibit growth of one of these transformed cell lines,[46] but not to inhibit growth of other two.[3,45] Therefore, it seems uncertain whether bFGF acts as an autocrine factor for bFGF-producing tumor cells. Recently, Masuda *et al.*[32,47] showed that human epidermoid carcinoma cells, A431 cells, secreted a bFGF-like factor into protein-free medium, in which the cells grew as well as in medium containing serum. They also showed that exogenous addition of this bFGF-like factor did not affect cell growth.

The present results also suggest that bFGF-like factor produced by human cancer cells, HuCC-T1, is irrelevant to the growth of these cells.

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