Purification from a Human Hepatoma Cell Line of a Basic Fibroblast Growth Factor-Like Molecule That Stimulates Capillary Endothelial Cell Plasminogen Activator Production, DNA Synthesis, and Migration

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A 17,500-dalton protein which stimulates plasminogen activator production in cultured bovine capillary endothelial cells has been purified from a SK-Hep-1 human hepatoma cell lysate by using heparin affinity chromatography and fast protein-liquid ion exchange chromatography. The purified molecule stimulated plasminogen activator production in a dose-dependent manner between 0.01 and 1 ng/ml. It also stimulated collagenase synthesis, DNA synthesis, and motility in capillary endothelial cells in the same concentration range. This molecule was identified as a basic fibroblast growth factor-like molecule on the basis of its biological activity, its affinity for heparin-Sepharose, and its cross-reactivity with a polyclonal antibody raised against the human placental basic fibroblast growth factor.

Neovascularization occurs in the microvasculature in response to angiogenic stimuli and is marked by the destruction of the blood vessel basal lamina, followed by migration and proliferation of the endothelial cells (1). Angiogenic preparations from a number of cells and tissues have been reported to induce a phenotype in cultured capillary endothelial cells which consists of increased mitosis (5, 6, 21, 22), chemotactic movement (2, 16, 24), and production of proteases (12). Each of these responses has been used in assays for the detection of putative angiogenesis factors.

Recently, the ability to stimulate plasminogen activator (PA) and collagenase production in endothelial cells has been used as an assay in the purification of an angiogenesis factor from human placenta. The purified molecule, an 18,700-dalton protein, stimulates PA and collagenase production, cell replication, and chemotactic movement in cultured capillary endothelial cells, indicating that a single molecule is able to induce the complete "angiogenic phenotype" in the endothelial cell (15). Moreover, this molecule induces angiogenesis in vivo in the chicken chorioallantoic membrane (15). This factor has been identified as human basic fibroblast growth factor (FGF) on the basis of its affinity for heparin-Sepharose, cross-reactivity with antibodies to basic FGF, and amino acid sequence data. Indeed, the ability of basic FGF to induce angiogenesis in vivo has also been reported by others (6, 10, 20).

Angiogenic factors have been identified in preparations from solid tumors and from tumor cell lines (7; for a recent review, see also reference 8). In particular, the cell lysate of the human hepatoma cell line SK-Hep-1 has been shown to be strongly angiogenic when assayed on the chorioallantoic membrane (18, 24). The same preparation has also been shown to stimulate PA and collagenase production (12), growth (18), and motility (16, 24) in cultured endothelial cells. These data therefore suggest the presence of a basic

FGF-like molecule in this preparation. We have attempted to purify the protease-inducing factor present in the SK-Hep-1 cells and to evaluate its identity with basic FGF.

MATERIALS AND METHODS

Cell culture. Bovine capillary endothelial (BCE) cells were isolated from bovine adrenal cortex as described previously (9). Once cultures were established, cells were grown in alpha minimal essential medium (aMEM) containing 5% (vol/vol) calf serum (CS) on gelatin-coated dishes. Human hepatoma SK-Hep-1 cells (obtained from J. Fogh, Sloan-Kettering Institute for Cancer Research, Rye, N.Y.) were grown in Eagle minimal essential medium with 15% fetal calf serum (FCS) and supplemented with nonessential amino acids (Flow Laboratories).

PA assay. Confluent cultures of BCE cells that had been maintained for at least 2 days in aMEM with 5% CS were changed to fresh medium containing the material to be tested. After incubation at 37°C for 16 h, the cell layers were washed twice with cold phosphate-buffered saline (PBS), pH 7.5, and were extracted with 0.5% (vol/vol) Triton X-100 in 0.1 M sodium phosphate, pH 8.1, and the cell extracts were assayed for PA activity as described previously (11).

Purification of the PA-inducing factor. All the purification steps, except the fast protein-liquid chromatography (FPLC) steps, were performed at 4°C. SK-Hep-1 cells from confluent monolayers were scraped into cold PBS and pelleted by centrifugation at $400 \times g$ for 10 min. The cell pellet was suspended in 10 volumes of PBS-0.5 M NaCl and sonicated for 3 min at 50 W with a Branson sonicator (Plainview, N.Y.). The extract was centrifuged ($10,000 \times g$, 1 h), and the supernatant was collected. The pellet was suspended in 1 volume of PBS-0.5 M NaCl, sonicated, and centrifuged. The two supernatants were pooled and passed through a column (28 by 75 mm) of heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with PBS-0.5 M NaCl. The column was washed with 0.5 M NaCl-3 mM EDTA-100 mM Tris, pH 7.5, and eluted with a 0.5 to 2 M NaCl gradient in the same buffer. Fractions were assayed for PA-inducing

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activity, and the active fractions were pooled and diluted with 3 mM EDTA–20 mM Tris, pH 7.5, until the conductivity was 20 mmho. The active material was then passed through a second heparin-Sepharose column (10 by 75 mm) equilibrated with 0.5 M NaCl in 3 mM EDTA–20 mM Tris, pH 7.5. The column was washed first with 0.5 M NaCl and then with 0.9 M NaCl, both in 3 mM EDTA–20 mM Tris, pH 7.5, and was eluted with a 0.9 to 2 M NaCl gradient in the same buffer. The active fractions were concentrated on a third heparin-Sepharose column (7 by 85 mm), which was washed with 0.5 M NaCl and then eluted with 2 M NaCl, both in 20 mM morpholinoethane sulfonate (MES), pH 6.0. The active fractions from the third heparin-Sepharose column were diluted 1:10 with 20 mM MES, pH 6.0, and the solution was clarified by centrifugation at $100,000 \times g$ for 1 h. The sample was then loaded on a Mono-S FPLC column (Pharmacia) equilibrated with 0.2 M NaCl–20 mM MES, pH 6.0. The column was washed with 0.2 M NaCl, and elution was achieved with a multilinear gradient of NaCl (0.2 to 2 M in 20 mM MES, pH 6.0). The fractions were assayed for PA-inducing activity, and the active fractions were pooled. Purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE. SDS-polyacrylamide gels (3% stacking gels and 12% resolving gels) were prepared and run as described previously (14). Proteins were detected with the silver stain procedure (23).

Protein determination. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.

DNA synthesis assays. The purified hepatoma factor was tested for its ability to stimulate DNA synthesis in BCE cells and BALB/c mouse 3T3 cells. Confluent cultures of BCE cells were maintained in aMEM with 5% CS for 7 days. The medium was then replaced with fresh aMEM with 5% CS containing various concentrations of purified hepatoma factor. After 20 h, the medium was replaced with Dulbecco modified Eagle medium (DMEM) containing 5% CS and 0.3 μ Ci of [125 I]iododeoxyuridine (125 IdU; 2,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. After 16 h, the cells were washed with cold PBS, and the incorporation of 125 IdU into acid-soluble material was determined as described previously (19). Stimulation of [3 H]thymidine incorporation into the DNA of monolayers of BALB/c 3T3 cells was determined as described previously (5).

Migration assay. Migration assays were performed in 200- μ l blind wells (Nucleopore Corp., Pleasanton, Calif.) with 5- μ m-pore-size polycarbonate polyvinylpyrrolidone-free filters coated with gelatin and fibronectin (4). Serial dilutions of the purified hepatoma factor in aMEM with 0.5% FCS were placed in the bottom wells. The filters were then inserted, and 5×10^4 BCE cells in 200 μ l of aMEM with 0.5% FCS were added to the upper wells. After a 4-h incubation at 37°C, the medium in the top wells was removed, and the cells on the upper surface of the filters were scraped off with a cotton swab. The filters were removed, dried at room temperature, and stained with Wright-Geimsa stain. The total number of cells on the lower filter surface was counted under a light microscope at 400 $\times$ magnification.

Inhibition of hepatoma factor activity by antiplacental basic FGF antibodies. Polyclonal antibodies against placental basic FGF were raised in rabbits by lymph node injection followed by intradermal boosts. These antibodies do not cross-react with other heparin-binding proteins on Western blot analysis (unpublished data). Ammonium sulfate (50%) cuts of both

immune and nonimmune sera (15.5 mg of protein per ml) were used in all experiments. Pure placental basic FGF and hepatoma-derived factor (1.5 ng/ml) were each incubated in aMEM with 5% CS and different dilutions of immune or nonimmune gamma globulins for 2 h at room temperature, and then the incubation mixtures were added to confluent BCE cells. Following overnight incubation, BCE cells were assayed for PA activity as described above.

Immunoprecipitation. For biosynthetic labeling of the hepatoma-derived factor, cultures of SK-Hep-1 cells were pre-incubated for 1 h in Eagle minimal essential medium (MEM) with 1% CS and 1/5 the standard concentrations of amino acids. Then the cultures were incubated overnight in fresh medium of the same type containing 20 μ Ci (300 Ci/mmol) of [35 S]cystine (New England Nuclear Corp.) per ml. At the end of this period, the conditioned medium was collected. The cells were washed twice with cold PBS, scraped from the plate with a rubber policeman, sonicated at 50 W for 30 s in PBS, and centrifuged for 1 min in an Eppendorf centrifuge. Both the conditioned medium and the cell lysate were loaded on 0.2-ml heparin-Sepharose columns equilibrated in PBS. The columns were washed with 0.5 M NaCl and eluted with 2 M NaCl in PBS. Immunoprecipitation was then performed on these eluates. Before immunoprecipitation, the samples (0.5 ml) were incubated with 50 μ l of nonimmune gamma globulins in 0.5 ml of radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 50 mM Tris hydrochloride, pH 7.4, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride) overnight at 4°C. Then 50 μ l of packed protein A-Sepharose beads (Sigma) were added, the samples were agitated at 4°C for 2 h, and the protein A-Sepharose beads were collected by centrifugation in an Eppendorf centrifuge for 1 min. The supernatants were divided in two portions (0.5 ml each) and used for specific immunoprecipitation. A 50- μ l amount of either antiplacental basic FGF gamma globulins or nonimmune gamma globulins was added to each portion, and the samples were incubated for 4 h at 4°C. Then 25 μ l of protein A-Sepharose was added, the samples were agitated for 2 h at 4°C, and the beads were collected by centrifugation. The beads were then washed five times with 1 ml of RIPA buffer and twice with 1 ml of water and suspended in a buffer containing 5% (vol/vol) 2-mercaptoethanol, boiled for 2 min, and loaded on an SDS–12% polyacrylamide gel. The gels were processed for fluorography, and the dried 2,5-diphenyloxazole (PPO)-impregnated gels were exposed to Kodak XAR-5 film for 3 weeks at –70°C.

Synthesis of urokinase and collagenase. BCE cells were grown to confluence on 35-mm dishes in aMEM with 5% CS. The medium was replaced with fresh aMEM with 5% CS containing no additions, hepatoma factor (2 ng/ml), or placenta factor (10 ng/ml). After 15 h at 37°C, the medium was changed to MEM containing 1/5 the standard concentration of amino acids with 1% CS and 50 μ Ci of [35 S]methionine (29.6 TBq/mmol; New England Nuclear) per ml. After another 8 h at 37°C, the medium was collected, the cells were washed once with PBS, and the washes were added to the collected media. The cells were extracted in RIPA buffer and scraped from the dish with a rubber policeman. Both conditioned medium and cell extracts were then immunoprecipitated with rabbit antiserum against human urokinase (a gift from Liliana Ossowski, The Rockefeller University, New York, N.Y.) or with serum from nonimmune rabbits as described above. The supernatants were used for a second immunoprecipitation with rabbit antiserum against human collagenase (a gift from Howard Welgus, Washington Uni-

FIG. 1. (A) Heparin-Sepharose 1. Hepatoma cell lysate (ca. 2 g) was loaded on a heparin-Sepharose column equilibrated with 0.5 M NaCl in PBS buffer. The column was washed with 0.5 M NaCl in 3 mM EDTA–20 mM Tris, pH 7.5, and eluted with a 0.5 to 2 M NaCl gradient in the same buffer. Fractions were tested for their ability to stimulate PA production by BCE cells. The material pooled for subsequent purification is indicated by the horizontal striped bar. (B) Heparin-Sepharose 2. The active fractions from the previous column were loaded on a second, smaller heparin-Sepharose column. The column was eluted sequentially with 0.5 M NaCl, 0.9 M NaCl, and a 0.9 to 2 M NaCl gradient in 3 mM EDTA–20 mM Tris, pH 7.5. Fractions were assayed as for panel A. (C) FPLC Mono-S. The active fractions from heparin-Sepharose 2 were concentrated on a third, smaller heparin-Sepharose column. The 2 M NaCl eluate from this column was run on an FPLC Mono-S column equilibrated with 0.2 M NaCl in 20 mM MES, pH 6.0. The column was eluted with a multilinear NaCl gradient, and the fractions were assayed as for panel A. The material pooled for SDS-PAGE analysis and biological studies is indicated by the striped horizontal bar.

versity Medical Center, St. Louis, Mo.) or with serum from nonimmune rabbits. The immunoprecipitates were run on SDS-PAGE, and the gels were processed for fluorography. The autoradiograms developed from these gels were scanned with a soft-laser scanner, and the area under the optical density peaks corresponding to urokinase and collagenase was determined.

RESULTS

Purification of the PA-stimulatory factor. Preliminary experiments demonstrated that, like the human placental basic FGF, the protease-inducing activity from the SK-Hep-1 cell lysate was heat sensitive and nondialyzable and eluted on gel filtration chromatography with an apparent molecular weight of approximately 20,000 (data not shown). Because basic FGF has a high affinity for heparin (6), we used heparin affinity chromatography as a first step in the purification of the hepatoma factor. When the hepatoma lysate was passed over a heparin-Sepharose column, all the PA-inducing activity bound to the column and was eluted at ca. 1.4 M NaCl (Fig. 1A). This afforded a 500-fold purification without any loss of activity (Table 1). The active fractions were bound to a second smaller heparin-Sepharose column which, before being eluted with an NaCl gradient, was washed with 0.9 M NaCl (Fig. 1B). This step resulted in a further threefold purification. However, almost 60% of the activity was lost in this step. The reasons for this loss are unknown. The active material was then loaded on a 3-ml heparin-Sepharose column which was eluted with a small volume of 2 M NaCl; this resulted in the concentration of the active material and in a further fourfold purification without any significant loss of activity. Since the placental basic FGF bound to carboxymethyl-Sephadex, we decided to use the cation exchange Mono-S FPLC column in the purification of the hepatoma-derived factor. As shown in Fig. 1C, when the active material from the third heparin-Sepharose column was loaded on a Mono-S column, almost 98% of the protein did not bind to the resin, but the PA-inducing activity bound and eluted as a single symmetric peak at 0.4 to 0.6 M NaCl. Analysis of the active fraction by SDS-PAGE showed the presence of a single band with a molecular weight of 17,500 under reducing (not shown) and nonreducing conditions (Fig. 2). The hepatoma-derived factor is, therefore, approximately 1,000 daltons smaller than the placental basic FGF (15).

Biological activity of the purified hepatoma factor. The purified molecule stimulated PA activity in BCE cells in a dose-dependent manner, half-maximal stimulation occurring at a concentration of 0.2 ng/ml (Fig. 3). Overnight incubation of BCE cells with the hepatoma factor at 1 ng/ml resulted in alteration of the cells from their typical cobblestone appearance to a more elongated, spindle-shaped morphology (results not shown). The same morphological alterations were previously observed in BCE cells treated with placental basic FGF (unpublished observations) or with 12-*O*-tetradecanoyl-phorbol 13-acetate (11).

To determine whether the increased PA activity stimulated by purified hepatoma factor reflected an increase in synthesis of PA, the ability of stimulated and unstimulated

TABLE 1. Purification and recovery of PA-stimulating factor from SK-HEP-1 cells

Prepn	Protein recovery (mg)	Activity recovery		Sp act (U/μg)	Purification (fold)
		U	%		
Cell lysate	1,955	87,500	100	0.05	1
Heparin-Sepharose 1	3.7	86,000	98	23.24	517
Heparin-Sepharose 2	0.39	24,333	28	62.39	1,386
Heparin-Sepharose 3	0.10	20,000	23	200	4,444
FPLC Mono-S	0.0015 ^a	11,667	13	7,778	173,000

^a Calculated from absorbance at 280 nm, assuming an extinction coefficient of $A_{280} = 0.6$ mg/ml, as determined for human placental basic FGF.

FIG. 2. SDS-PAGE. Ca. 100 ng of purified hepatoma-derived factor was run on a 12% polyacrylamide gel in nonreducing conditions. Proteins were visualized with silver staining. Molecular weight standards were phosphorylase A (90,000), bovine serum albumin (68,000), aldolase (45,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), myoglobin (17,000), and lactalbumin (14,200). Standards are indicated in thousands on the figure.

BCE cells to incorporate [^{35}S]methionine into immunoprecipitable urokinase-type PA was compared. Untreated BCE cells and cells stimulated with purified hepatoma factor (2 ng/ml) were incubated for 8 h in medium containing [^{35}S]methionine, and the conditioned medium and extracts of these cells were immunoprecipitated with antibodies to urokinase. When the immunoprecipitated material was run on SDS-PAGE, about four times more radioactivity was detected in the bands corresponding to bovine urokinase

FIG. 3. Effect of the purified hepatoma-derived factor on PA production in BCE cells. Confluent cultures of BCE cells were given fresh medium containing the indicated concentrations of the purified hepatoma factor. After 24 h of incubation at 37°C, the cells were extracted and extracts were assayed for PA.

TABLE 2. Stimulation of urokinase and collagenase synthesis by purified hepatoma factor and purified placenta factor^a

Treatment (ng/ml)	Relative density of immunoprecipitated bands on autoradiography	
	Urokinase	Collagenase
None	1.0	1.0
Hepatoma factor (2)	3.4	2.9
Placenta factor (10)	3.8	2.6

^a Confluent cultures of BCE cells on 35-mm dishes were incubated in fresh aMEM with 5% CS and the above additions for 15 h. The cells were then incubated in labeling medium with the same additions for an additional 8 h. The conditioned medium and extracts of the cells from these cultures were then immunoprecipitated with antibodies to urokinase and antibodies to collagenase. The immunoprecipitated material was run on SDS-PAGE, and the dried gel was exposed for autoradiography. The autoradiograms were scanned with a soft-laser scanner, and the optical density of the bands corresponding to urokinase and to collagenase in the treated cultures were compared with the corresponding bands from the untreated culture (assigned a value of 1.0).

in cells stimulated with hepatoma factor than in unstimulated cells (Table 2). An equivalent stimulation was obtained with placenta factor in these cells (Table 2). Thus, the increase in PA activity reflects an increase in the amount of urokinase synthesized.

Since the FGF-like molecule purified from placenta stimulated both PA and collagenase production (15), the effect of the purified hepatoma factor on collagenase production was investigated. The conditioned medium and cell extracts used above were immunoprecipitated with antibodies to interstitial collagenase, and the immunoprecipitated material was run on SDS-PAGE. Cells stimulated with hepatoma factor contained about three times more radioactivity in the bands corresponding to interstitial collagenase than did unstimulated cells (Table 2). A similar stimulation was obtained with placenta factor in these cells (Table 2). These results demonstrate that purified hepatoma factor stimulates the synthesis of both PA and collagenase in BCE cells.

The pure hepatoma factor, like the placenta factor (15), was also able to stimulate DNA synthesis in BCE cells. As shown in Fig. 4, addition of the hepatoma factor to confluent BCE cells stimulated the incorporation of ^{125}I dU into DNA in a dose-dependent manner at concentrations similar to those found to stimulate PA activity. This stimulation was

FIG. 4. Effect of the purified hepatoma-derived factor on DNA synthesis in BCE cells. Confluent cultures of BCE cells were incubated for 20 h with the indicated concentrations of the purified hepatoma factor and were then labeled for 16 h with ^{125}I dU. The amount of ^{125}I dU incorporated into trichloroacetic acid-insoluble material was then measured.

FIG. 5. Effect of the purified hepatoma-derived factor on DNA synthesis in BALB/c 3T3 mouse cells. Quiescent cultures of BALB/c 3T3 mouse cells were incubated in DMEM with 0.4% CS containing [³H]thymidine and the indicated concentrations of the purified hepatoma factor. After 24 h, the amount of radioactivity incorporated in the trichloroacetic acid-precipitable material was measured.

paralleled by an increase in the amount of protein in the cell monolayer (data not shown), indicating that the stimulation of ¹²⁵IIdU incorporation reflects stimulation of BCE cell growth and not simply of DNA synthesis. To confirm the growth-stimulatory activity of the hepatoma-derived factor, the ability of this molecule to stimulate DNA synthesis in BALB/c 3T3 cells was investigated. As shown in Fig. 5, [³H]thymidine incorporation into the DNA of these cells was stimulated in a dose-dependent manner at concentrations similar to those that were effective in BCE cells.

When the ability of the hepatoma factor to stimulate the motility of BCE cells was investigated, the molecule increased cell motility in the modified Boyden chamber assay at concentrations ranging from 1 to 10 pg/ml (Fig. 6). Thus, the hepatoma-derived protein, like the placental basic FGF (15), stimulates protease synthesis, cell replication, and motility in cultured BCE cells. In all the experiments performed, the placental basic FGF was used as a positive control; the degree of maximal stimulation induced by the hepatoma factor was similar to that induced by the placental basic FGF for all parameters tested (results not shown).

Immunological characterization of the purified hepatoma factor. We investigated whether polyclonal antibodies raised against the human placental basic FGF cross-react with the purified hepatoma factor. For this purpose, equal amounts of the two factors were incubated with dilutions of the antibodies and then assayed for PA-stimulatory activity on BCE cells. The antibodies quenched the activity of both factors in a similar dose-dependent manner, indicating that the two molecules are immunologically related (Fig. 7). The antiplacental basic FGF antibodies, as well as a polyclonal antiserum raised against the amino-terminal sequence of bovine brain basic FGF (3), were also shown to cross-react with the hepatoma factor in an immunoblot assay (data not shown), confirming the immunological relationship between the hepatoma-derived factor and basic FGF.

To demonstrate that the hepatoma factor was actually

synthesized by the hepatoma cells, SK-Hep-1 cells were labeled overnight with [³⁵S]cystine. Both the cell lysate and the conditioned medium were independently partially purified on a 0.2-ml heparin-Sepharose column, and the 2 M NaCl eluates were immunoprecipitated with anti-placental basic FGF antibodies. Analysis of the immunoprecipitates by SDS-PAGE showed in the cell lysate a major radioactive band with a molecular weight of 17,500, identical in size to the purified hepatoma factor. After longer exposure, two minor bands of 19,000 and 23,000 daltons were observed. No radioactive bands were detected in the conditioned medium (Fig. 8).

DISCUSSION

In this paper we describe the isolation and partial characterization of a basic FGF-like molecule from the human hepatoma SK-Hep-1 cell line. This protein stimulates PA production, cell division, and cell migration in cultured capillary endothelial cells at less than 1 ng/ml. This represents the first description of the isolation of a protein with all of these activities from a human tumor cell line.

The protein isolated from the SK-Hep-1 cells has been identified as a basic FGF-like molecule on the basis of the following observations. (i) The purification scheme adopted here is very similar to that which we used previously to purify human placental basic FGF (15). Of particular interest, the hepatoma factor, like all basic FGF-like molecules purified so far (3, 6, 21), bound tightly to heparin-Sepharose and eluted at an NaCl concentration of approximately 1.4 to

FIG. 6. Effect of the purified hepatoma-derived factor on motility of BCE cells. The experiment was performed in Boyden blind well chambers. Medium containing 0.5% FCS and the indicated concentrations of the purified factor was placed in the lower chambers. BCE cells (5×10^4) in medium containing 0.5% FCS were placed in the upper chambers. After a 4-h incubation, the cells on the lower surface of the filter separating the two chambers were counted. Vertical lines indicate standard errors.

1.5 M. (ii) The hepatoma factor showed the same biological activity as the human placental basic FGF and similar potency. (iii) The activity of the purified molecule was inhibited by polyclonal antibodies raised against the human placental basic FGF. The hepatoma protein also cross-reacted with antiserum prepared against a synthetic peptide representing the amino-terminal sequence of human brain basic FGF. (iv) Immunoprecipitation experiments with radiolabeled SK-Hep-1 cells have shown that the anti-placental basic FGF antibodies cross-react with a molecule of the same molecular weight as the purified hepatoma factor, indicating that this protein is synthesized by the cell and does not derive from the serum present in the culture medium.

The hepatoma factor seems to be slightly smaller than the placental basic FGF (17,500 versus 18,700 daltons). Heterogeneity in molecular weight of bovine basic FGF purified from different sources has been already reported and seems to depend on the cleavage of amino acids from the amino-terminal portion of the molecule (6). The small differences in molecular weight observed for basic FGF-like molecules may be explained by differences in the extraction procedure, which can cause the cleavage of peptide fragments of different length. On the other hand, it is interesting that two proteins with molecular weights greater than that of the hepatoma factor were detected in the immunoprecipitate of the SK-Hep-1 cell lysate. These two molecules, 19,000 and 23,000 daltons, were present in lower amounts than the 17,500-dalton form and might represent precursors of the purified hepatoma factor.

It is also interesting that the immunoprecipitation experiments indicate that no protein is released from the hepatoma cells into the culture medium. Indeed, no PA-stimulatory activity was detectable in the conditioned medium of these cells, even after concentration on a heparin-Sepharose column (D. Moscatelli, M. Presta, J. Joseph-Silverstein, and D. B. Rifkin, *J. Cell. Physiol.*, in press). We have recently

FIG. 7. Inhibition of the hepatoma factor and of the human placental basic FGF PA-stimulatory activity by anti-placental basic FGF antibodies. Pure placental basic FGF and hepatoma factor (1.5 ng/ml) were incubated with dilutions of immune or nonimmune gamma globulins for 2 h at room temperature and then added to confluent BCE cells. Following overnight incubation, BCE cells were assayed for PA activity. Symbols: ●,▲, placental basic FGF; ○,△, hepatoma factor; ●,○, immune gamma globulins; ▲,△, nonimmune gamma globulins.

FIG. 8. Immunoprecipitation of biosynthetically labeled basic FGF from the SK-Hep-1 cells. Confluent cultures of SK-Hep-1 cells were incubated for 16 h in MEM with 1% CS and 1/5 the standard concentrations of amino acids, containing 20 μ Ci of [³⁵S]cystine per ml. The cell lysate and the conditioned medium were concentrated on a 0.2-ml heparin-Sepharose column as described in Materials and Methods. The 2 M NaCl eluates were immunoprecipitated with polyclonal antibodies raised against the human placental basic FGF as described in Materials and Methods. The immunoprecipitates were loaded on an SDS-12% polyacrylamide gel in reducing conditions, and the gel was processed for fluorography and exposed for 3 weeks at -70° C. NI, Nonimmune gamma globulins; I, immune gamma globulins. Molecular weights (in thousands) are indicated to the left.

found that the lack of release of basic FGF is a property shared by many cell types that produce basic FGF and is not limited to SK-Hep-1 cells (Moscatelli et al., in press). These observations represent the first attempt to examine intracellular versus extracellular levels of basic FGF, in contrast to examining the level of basic FGF in extracts of whole tissue, and point out a critical step in the regulation of basic FGF levels in the organism: the control of its release from the cell. Since basic FGF is believed to function via high-affinity membrane receptors (17), these results suggest that the mechanisms controlling basic FGF release may be crucial to the modulation of its action. It is possible that the release of basic FGF is controlled by specific environmental factors such as pO_2 , proteolytic enzymes, or macrophage- and lymphocyte-derived factors.

The identification of a human tumor cell line producing basic FGF in vitro will allow the study of how the synthesis, processing, and secretion of this molecule are regulated. Moreover, the SK-Hep-1 cells can be used as a source of basic FGF mRNA, making the cloning of its gene more feasible. Preliminary experiments indicate that this is indeed possible.

Since this work was submitted, Klagsbrun et al. (13) have also reported the synthesis of a basic FGF-like molecule by SK-Hep-1 cells.

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