

Human tumor cells synthesize an endothelial cell growth factor that is structurally related to basic fibroblast growth factor

(heparin affinity/angiogenesis)

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ABSTRACT A human hepatoma cell line synthesizes, as evidenced by metabolic labeling, an endothelial cell mitogen that is found to be mostly cell associated. The hepatoma-derived growth factor (HDGF) has been purified to homogeneity by a combination of Bio-Rex 70, heparin-Sepharose, and reverse-phase chromatography; it is a cationic polypeptide with a molecular weight of about 18,500-19,000. HDGF is structurally related to basic fibroblast growth factor (FGF). Immunological analysis demonstrates that antiserum prepared against a synthetic peptide corresponding to the amino-terminal sequence of basic FGF cross-reacts with HDGF when analyzed by electrophoretic blotting and by immunoprecipitation. Sequence analysis of tryptic fragments demonstrates that HDGF contains sequences that are homologous to both amino-terminal and carboxyl-terminal sequences of basic FGF.

Endothelial cell growth factors have been isolated from a variety of tissue sources. The angiogenic activity of these polypeptides suggests that they may play an important role in the vascularization of tissues. Recent studies indicate that possibly all endothelial cell growth factors have a strong affinity for heparin (1-7). Heparin affinity chromatography has so greatly facilitated the purification of endothelial cell growth factors to homogeneity (1, 2, 4, 7) that structural comparison of different endothelial cell growth factors is now possible. Of particular interest is the question of what is the relationship between endothelial cell growth factors found in tumors and in normal tissue. Are tumor-derived endothelial cell growth factors tumor-specific proteins or are they normally occurring proteins that are being expressed by the tumor in an abnormal manner?

This report analyzes the structural relationships of a heparin-binding endothelial cell growth factor isolated from tumor cells with one isolated from normal brain. To undertake structural studies, we have purified and partially sequenced a hepatoma-derived growth factor (HDGF) that is synthesized by a human hepatoma cell line. We have also prepared antiserum directed against a synthetic peptide corresponding to the amino-terminal sequence of basic fibroblast growth factor (FGF), an endothelial cell growth factor purified from bovine (2, 8) and human (9) brain. The total amino acid sequence of bovine brain basic FGF has been recently determined (10). In this report we present evidence that HDGF and FGF are structurally related by demonstrating (i) that anti-FGF antiserum cross-reacts with HDGF and (ii) that HDGF contains at least two peptide sequences whose primary structures are homologous to peptide sequences found in pituitary FGF.

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METHODS AND MATERIALS

Hepatoma Cell Culture. The human hepatoma cell line SK-HEP-1 (11) was grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (Colorado Serum, Denver, CO). For small-scale culture, SK-HEP-1 cells were grown in monolayer. For large-scale culture, SK-HEP-1 cells were grown in suspension in 100-liter Vibromixer reactors (12) to produce about 5×10^{10} cells.

Metabolic Labeling. Two confluent T 150 flasks (Costar, Cambridge, MA) of SK-HEP-1 cells (about 8×10^7 cells) were incubated for 24 hr in 10 ml each of methionine-free HB-101 medium (Hana Biologicals) supplemented with 2% fetal bovine serum and [35 S]methionine (Amersham; 680 Ci/mmol; 1 Ci = 37 GBq) at 50 μ Ci/ml.

Growth Factor Analysis. HDGF activity was assayed as previously described by measuring the ability of samples to stimulate DNA synthesis in BALB/c 3T3 cells (13) and to stimulate the proliferation of capillary endothelial cells (1, 3).

Extraction of Hepatoma Cells. SK-HEP-1 cells were harvested from monolayer culture by trypsinization and from suspension cultures by centrifugation. The cells were washed with Dulbecco's phosphate-buffered saline (GIBCO), resuspended in 1 M NaCl/0.01 M Tris-HCl, pH 7.5 ($1-4 \times 10^7$ cells per ml), and disrupted by sonication (Sonic and Materials, Danbury, CT) or homogenization (Waring blender) for 1 min. After a centrifugation at $25,000 \times g$ for 30 min, the pellet was discarded and the supernatant was dialyzed overnight without any changes against 0.01 M Tris-HCl, pH 7.5, so as to lower the concentration of NaCl to 0.15 M and stored at 4°C.

Column Chromatography. (i) Bio-Rex 70 cation-exchange chromatography. For large-scale preparations (about 5×10^{10} cells), dialyzed hepatoma cell extract (1250 ml, 4×10^6 units) was mixed with 1750 ml of Bio-Rex 70 (Bio-Rad, 200-400 mesh) equilibrated with 0.15 M NaCl/0.01 M Tris-HCl, pH 7.5, by stirring overnight at 4°C. The Bio-Rex 70 was poured into a column (5×100 cm) and washed with 2000 ml of equilibration buffer. Growth factor activity was eluted by batch with 2000 ml of 0.6 M NaCl/0.01 M Tris-HCl, pH 7.5, at a flow rate of 60 ml/hr at 4°C. (ii) Heparin-Sepharose chromatography. Growth factor, prepared by Bio-Rex 70 chromatography (300 ml, 1.5×10^6 units) was applied directly to a column of heparin-Sepharose (Pharmacia, 2×13 cm, 40 ml) equilibrated with 0.6 M NaCl/0.01 M Tris-HCl, pH 7.5. After a wash of about 5 column volumes, growth factor was eluted with a gradient (400 ml) of 0.6-3.0 M NaCl in 0.01 M Tris-HCl, pH 7.5, at a flow rate of 40 ml/hr at 4°C. (iii) Reverse-phase chromatography. Growth factor was analyzed by reverse-phase liquid chromatography (RPLC) using a Beckman model 334 HPLC gradient system and an RPSC

Abbreviations: HDGF, hepatoma-derived growth factor; FGF, fibroblast growth factor; RPLC, reverse-phase HPLC.

C3 column (Beckman, 0.46 × 7.5 cm). The samples were applied via a separate minipump (LDC/Miniroy, LDC/Milton Roy, Riviera Beach, CA) at a flow rate of 3 ml/min at room temperature. After a wash with 0.1% trifluoroacetic acid, a 0–60% linear gradient (120 ml) of acetonitrile/2-propanol (50:50, vol/vol) in 0.1% trifluoroacetic acid was applied to the column at a flow rate of 1.0 ml/min and fractions (1 ml) were collected. For measurements of biological activity, aliquots from each fraction were quickly diluted 20-fold into phosphate buffered saline supplemented with serum albumin at 1 mg/ml to bring the pH to neutrality.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (NaDodSO₄/PAGE). Samples were analyzed by NaDodSO₄/PAGE as described by Laemmli (14) and the polypeptide bands were visualized by silver stain (15).

Preparation of Synthetic Peptides. A peptide corresponding to the amino-terminal 15 amino acids of bovine FGF (2, 8) was synthesized by solid-phase methods (16, 17), using an automated Applied Biosystems 430 A peptide synthesizer.

Production of Polyclonal Antibodies. The synthetic FGF peptide was conjugated to keyhole limpet hemocyanin (KLH) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (18). Rabbits were immunized by multisite intradermal injections of KLH-peptide conjugate (500 μg) emulsified with complete Freund's adjuvant, followed 3 and 6 weeks later with a subcutaneous injection of 200 μg of KLH-peptide conjugate emulsified in incomplete Freund's adjuvant. The titer of the antisera after the second booster injection was about 1:20,000–1:90,000 as determined in an ELISA using unconjugated peptide as the antigen.

Protein Blot Analysis. Protein was electrophoresed on NaDodSO₄/18% polyacrylamide gels and transferred electrophoretically to BA-83 nitrocellulose paper (Trans-blot cell, Bio-Rad) by using previously described methods (19). The nitrocellulose paper was either stained for protein with Aurodye colloidal gold reagent (Janssen Life Sciences Products, Beerse, Belgium) or incubated with anti-FGF antiserum and visualized by successive incubations with biotinylated goat anti-rabbit antibodies, peroxidase-conjugated streptavidin, and 4-chloro-1-naphthol substrate (20).

Immuno-Dot Analysis. Samples were filtered through a 96-well microfiltration manifold (Bio-dot apparatus, Bio-Rad) onto BA-83 0.2-μm-pore nitrocellulose sheets (21) and incubated with anti-FGF antiserum (1:1500) to form immunodots that were visualized as described for the protein blot assay. The intensity of the immuno-dots was quantitated by reflectance densitometry using a Hoefer instrument (Hoefer Scientific Instruments, San Francisco).

Immunoprecipitation. Cells labeled with [³⁵S]methionine were extracted with 4–5 ml of phosphate-buffered saline supplemented with 0.5% Triton-X 100, 0.5% sodium deoxycholate, and 0.005 M phenylmethanesulfonyl fluoride (PBS-TD) for 4 hr at 4°C and centrifuged at 3000 × g for 10 min to remove debris. For immunoprecipitation, 150 μl of staphylococcal protein A-Sepharose was resuspended in 1 ml of PBS-TD and mixed with 30 μl of anti-FGF serum by shaking for 2 hr. The beads were washed and mixed with [³⁵S]methionine-labeled cellular extract (about 10 ml) supplemented with gelatin (final concentration 2 mg/ml) by shaking for 4 hr at 4°C. The antigen-antibody complex was extracted from the beads with 2 vol of Laemmli NaDodSO₄/PAGE sample gel buffer (14), boiled for 5 min, and analyzed by NaDodSO₄/PAGE and fluorography. Detection of bands on Kodak XAR-5 x-ray film was facilitated by impregnating the polyacrylamide gel with EN³HANCE (New England Nuclear).

Protein Sequence Analysis. HDGF was desalted by RPLC, dried in a Speedvac concentrator (Savant), resuspended (80 μg/0.2 ml) in 0.2 M ammonium bicarbonate buffer, and digested with trypsin (Cooper Biomedical, Malvern, PA,

treated with tosylamidophenylethyl chloromethyl ketone, enzyme-to-substrate ratio of 1:50, wt/wt) at 37°C for 8 hr. The digest was dried in a Speedvac concentrator, dissolved in 0.2 ml of 6 M guanidine-HCl/0.1% trifluoroacetic acid, and applied to a Vydac phenyl RPLC column (The Separations Group, Hesperia, CA, 0.46 × 25 cm). The tryptic fragments were separated with a linear gradient (120 ml) of 0–60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The peptide peaks were collected, dried in a Speedvac concentrator, dissolved in 0.1 ml of 0.5% trifluoroacetic acid/0.1% NaDodSO₄, applied to a cartridge filter treated with Polybrene, and analyzed with an Applied Biosystems (Foster City, CA) 470A sequencer (22).

RESULTS

Purification of HDGF. When the human hepatoma cell line SK-HEP-1 was grown in culture, the growth factor activity was found to be associated mostly with the cells (up to 90%) rather than with the conditioned medium. About 0.6–1.3 units of growth factor activity were found per 10⁴ cells. For large-scale purification, HDGF was isolated from lysates of SK-HEP-1 cells grown in suspension. In the first step, extracts of SK-HEP-1 cells were applied to a column of Bio-Rex 70 and HDGF was eluted with 0.6 M NaCl and applied directly to a column of heparin-Sepharose (Fig. 1 *Upper*). One peak of growth factor activity, as measured by the ability of fractions to stimulate both DNA synthesis in 3T3 cells and proliferation of capillary endothelial cells, eluted at about 1.6–1.8 M NaCl. Analysis of the active fractions by NaDodSO₄/PAGE and silver stain revealed the presence of a single but slightly diffuse polypeptide band with a molecular weight of about 18,500–19,000 (*Inset* of Fig. 1 *Upper*). HDGF had a specific activity of about 5–7 units/ng and stimulated both 3T3 and capillary endothelial cell proliferation at about 0.5–1.0 ng/ml.

HDGF purified by heparin-Sepharose affinity chromatography could be partially resolved into two superimposable peaks of 3T3 and capillary endothelial cell growth factor activity by RPLC (Fig. 1 *Lower*). When analyzed by NaDodSO₄/PAGE, a doublet with molecular weights of about 18,500 and 19,000 was found in each of the active fractions, one of which is shown in *Inset* of Fig. 1 *Lower*. As will be shown below in Fig. 3, both bands were positive in a protein blot analysis using the same antiserum. These results suggest that HDGF exists in structurally related multiple forms. As of yet, we have not been able to separate these two forms of HDGF.

Immunological Cross-Reactivity of HDGF and FGF. An antiserum was prepared against a synthetic peptide corresponding to the amino-terminal sequence of FGF (2, 8, 10). Fractions collected after RPLC (Fig. 1 *Lower*) were tested for the ability to cross-react with the anti-FGF antiserum in an immuno-dot assay (Fig. 2). Only those fractions containing biologically active HDGF were found to cross-react with anti-FGF. The ability of anti-FGF antiserum to cross-react with HDGF was further analyzed by use of an electrophoretic blot ("Western Blot") assay (Fig. 3 *Left*). Both crude and pure preparations of HDGF were electrophoresed on NaDodSO₄/polyacrylamide gels and the proteins were blotted onto nitrocellulose by electrophoretic transfer. HDGF, purified to a high degree as shown by the protein stain (lane 1), was clearly immunoreactive with anti-FGF antiserum (lane 3). The antiserum was also highly specific in its ability to detect HDGF in a partially purified preparation of hepatoma cells. Of the many proteins in the crude preparation (lane 2), only a polypeptide doublet with molecular weights of about 18,500 and 19,000 was clearly immunoreactive (lane 4). The immunoreactivity of the anti-FGF antiserum with

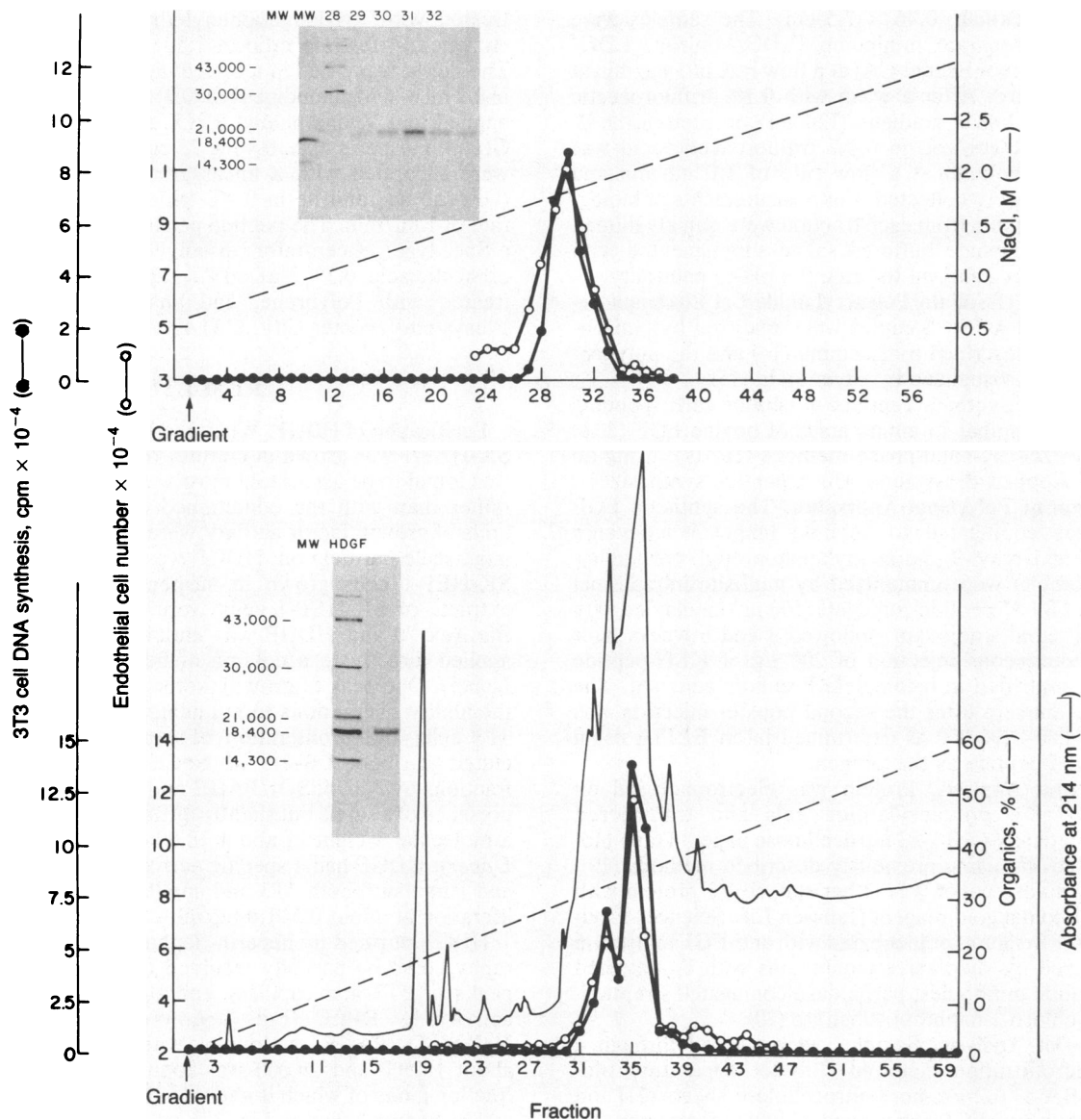


FIG. 1. Purification of HDGF. Purification by column chromatography was monitored by two assays: stimulation of DNA synthesis in 3T3 cells (●) and stimulation of the proliferation of capillary endothelial cells (○). (Upper) Extracts of SK-HEP-1 cells (about 5×10^{10} cells, 4×10^6 units) were mixed with Bio-Rex 70 and growth factor was eluted with 0.6 M NaCl. Active fractions (1.5×10^6 units) were applied directly to a column of heparin-Sepharose and growth factor was eluted with a gradient of 0.6–3.0 M NaCl. (Inset) Each of the active fractions (28–32) was dialyzed against distilled water, lyophilized, and analyzed by NaDodSO₄/PAGE (MW, molecular weight markers). (Lower) The remainders of fractions 28–31 (400,000 units) were pooled and applied to an HPLC C3 reverse-phase column. Growth factor was eluted with a 0–60% linear gradient of acetonitrile/2-propanol (50:50, vol/vol) in 0.1% trifluoroacetic acid. (Inset) Fractions 32–37 were analyzed by NaDodSO₄/PAGE. Each fraction displayed the electrophoretic pattern shown.

both bands of the doublet suggests the presence of microheterogeneity in HDGF preparations.

Immunoprecipitation. Anti-FGF antiserum could also be used to show that HDGF is biosynthesized by SK-HEP-1 cells (Fig. 3 Right). SK-HEP-1 cells were labeled in culture with [³⁵S]methionine. [³⁵S]Methionine-labeled hepatoma extracts were analyzed by NaDodSO₄/PAGE and fluorography directly (lane 2), after incubation with preimmune serum as a control (lane 3), and after incubation with anti-FGF antiserum (lane 4). A radioactive band, with a molecular weight of about 18,000–19,000 was precipitated by the anti-FGF antiserum but not by the preimmune serum. Thus, it appears that HDGF can be metabolically labeled. Several larger molecular weight bands were precipitated in a nonspecific manner with both the preimmune serum and the anti-FGF antiserum.

Sequence Homologies Between HDGF and FGF. It could be

concluded from the immunoreactivity experiments that HDGF and FGF are structurally related but not to what extent. Accordingly, an attempt was made to elucidate the partial amino acid sequence of HDGF. When 1.5 nmol (about 28 μg) of HDGF was submitted for sequence analysis, no sequence data could be obtained suggesting that HDGF was blocked at its amino-terminal amino acid. Since the amino-terminal sequences for both bovine and human FGF have been reported (2, 8–10), the block in HDGF suggested that the amino-terminal regions of HDGF and FGF were different. Subsequently, HDGF was cleaved with trypsin and the fragments were separated by RPLC and sequenced. One of the sequences was found to be (Thr)-Leu-Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Xaa-(Gly) (Fig. 4). Starting with its third amino acid residue (Pro), the sequence of this HDGF tryptic peptide was found to be identical with the amino-terminal sequence of bovine FGF and human FGF as well (Fig. 4). The

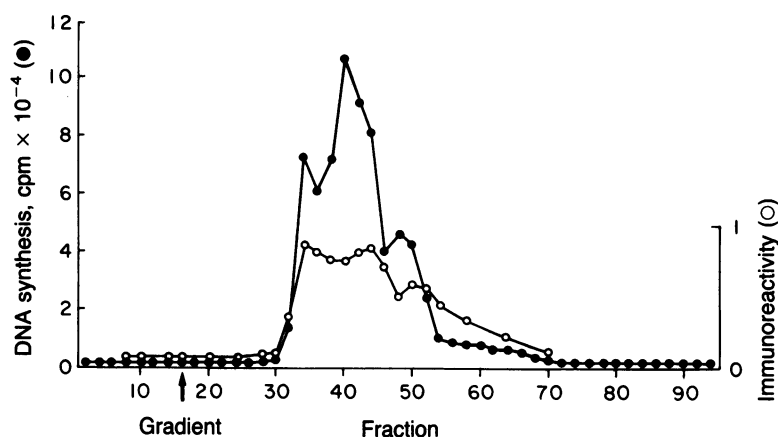


FIG. 2. Immunodot analysis of HDGF interaction with anti-FGF antiserum. HDGF was purified by RPLC as described in Fig. 1. Fractions eluting from the column were tested for growth factor activity (●) and for the ability to react with antiserum prepared against a synthetic peptide corresponding to the amino-terminal sequence of FGF in an immunodot assay (○). The reaction of HDGF with anti-FGF antiserum on nitrocellulose resulted in the formation of immunodots whose intensity was quantitated by reflectance densitometry. The intensity of dots is plotted on an arbitrary scale from 0 (maximal reflectance, no immunoreaction) to 1 (no reflectance, maximal immunoreaction).

sequence data suggest that HDGF contains within it the amino-terminal sequence of FGF. However, in HDGF, this sequence is internal and contains at least two additional amino acids on the amino-terminal side. Furthermore, the blocked nature of the HDGF amino terminus suggests that HDGF contains more than two additional acids that are amino-terminal to the amino-terminal sequence of FGF.

Another tryptic fragment of HDGF had the sequence Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-(Ser) (Fig. 4). This sequence is identical with amino acids 136–146, the carboxyl terminus of bovine FGF (10). Thus HDGF contains amino acid sequences homologous to both the amino and

carboxyl termini of basic FGF. However, at this time the positions of these sequences in HDGF are not known.

DISCUSSION

Human hepatoma cells in culture synthesize, as evidenced by metabolic labeling, a heparin-binding endothelial cell growth factor that is structurally related to bovine basic FGF. The evidence for this structural relationship is (i) that antiserum prepared against the amino-terminal sequence of FGF cross-reacts with HDGF in immunodot, electrophoretic blot, and immunoprecipitation assays and (ii) that HDGF contains amino acid sequences homologous to the amino-terminal and carboxyl-terminal regions of FGF. However, there are several important structural differences between HDGF and FGF. First of all, HDGF, with a reported molecular weight of 18,500–19,000, is larger than FGF, which has a molecular weight of 16,400 (10). Those molecular weight differences are evident when HDGF and basic FGF are analyzed on the same polyacrylamide gel (23). Second, HDGF appears to be blocked at its amino terminus, unlike either basic or acidic FGF (10, 24). We find that a chondrosarcoma-derived endothelial cell growth factor (1) is also blocked at the amino terminus. The possibility exists that blockage at the amino-terminal position is an important difference between endothelial cell growth factors in tumors and in normal tissue.

The exact extent of the homology between HDGF and FGF will not be known until the full sequence of HDGF is determined. One possibility arising from the sequencing data obtained so far is that HDGF and FGF have extensive sequence homology but that HDGF has an additional peptide domain on its amino terminus that is absent from FGF. The

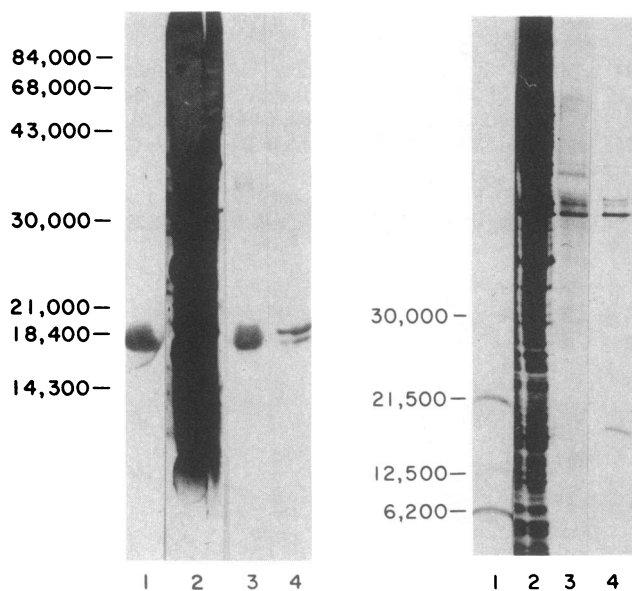


FIG. 3. Immunodetection of HDGF by using anti-FGF antiserum after NaDodSO₄/PAGE. (Left) Electrophoretic blot analysis. A crude preparation of HDGF, partially purified by Bio-Rex 70 chromatography, and HDGF purified to homogeneity as described in Fig. 1 were electrophoresed on NaDodSO₄/18% polyacrylamide gel. The proteins were transferred electrophoretically in replicate to nitrocellulose paper and either stained for protein or incubated with anti-FGF antiserum. Lane 1, purified HDGF stained for protein; lane 2, partially purified HDGF stained for protein; lane 3, purified HDGF stained with anti-FGF antiserum; lane 4, partially purified HDGF stained with anti-FGF antiserum. (Right) Immunoprecipitation. SK-HEP-1 cells were grown in the presence of [³⁵S]methionine, extracted, and analyzed by NaDodSO₄/PAGE and fluorography. Lane 1, ¹⁴C-labeled molecular weight standards; lane 2, SK-HEP-1 cell extract; lane 3, SK-HEP-1 extract incubated with preimmune serum; lane 4, SK-HEP-1 extract incubated with anti-FGF antiserum.

Bovine FGF N-terminal*	P A L P E D G G S G
HDGF tryptic peptide I [†]	(T) L P A L P E D G G X(G)

Bovine FGF C-terminal*	A I L F L P M S A K S
HDGF tryptic peptide II [†]	A I L F L P M S A K(S)

FIG. 4. Comparison of amino acid sequences in FGF and HDGF. The standard one-letter notation is used.

*The amino-terminal (residues 1–10) and carboxyl-terminal (residues 136–146) amino acid sequences of bovine FGF are those determined by Esch *et al.* (10). Human basic FGF has the same amino-terminal sequence as bovine FGF (9).

[†]About 3.2 nmol of HDGF was digested with trypsin and the tryptic peptides were separated by RPLC. About 50 pmol of HDGF tryptic peptide I and 1.3 nmol of HDGF tryptic peptide II were sequenced. The initial and repetitive yields were 50% and 83%, respectively, for HDGF tryptic peptide I and 50% and 87% for HDGF tryptic peptide II. An X indicates an unidentified amino acid residue. Parentheses surrounding an amino acid residue indicate that the residue was the predominant one in the degradation cycle.

amino terminus of FGF appears to be internal in HDGF by at least 2 amino acids, and preliminary evidence suggests possibly by as many as 15 amino acids. Such a structure would be consistent with the observation that the molecular weight of HDGF is larger than that of FGF by about 15–20 amino acids. The biosynthesis of FGF may be such that the extra amino-terminal domain found in HDGF is never synthesized or, alternatively, it is synthesized but is cleaved during processing of the growth factor. Another possibility is that the true amino-terminal portion of FGF (including an amino-terminal block) is cleaved and lost during the biochemical purification of FGF. The existence of a specific tumor sequence might be important in trying to differentiate between endothelial cell growth factors found in normal tissues and tumors.

The presence of an FGF-like growth factor in chondrosarcoma has been recently described by Baird *et al.* (25), who suggest that this FGF-like growth factor is probably identical to the chondrosarcoma-derived endothelial cell growth factor described by Shing *et al.* (1). Since chondrosarcoma-derived growth factor is angiogenic (26), as is HDGF (unpublished observations) and basic FGF isolated from pituitary (10), it appears that structurally similar proteins may be involved in tumor and normal angiogenesis. Thus, tumor-induced angiogenesis may result from the abnormal regulation of an otherwise normally occurring endothelial cell growth factor.

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