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Research Article

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Isolation, Characterization, and Localization of Heparin-binding Growth Factors in the Heart

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Abstract

Acidic and basic fibroblast growth factors (aFGF and bFGF) are angiogenic polypeptide mitogens for cells of mesodermal and neuroectodermal origin. In this report we describe the purification from several normal human hearts (including a very fresh, nonischemic sample) of heparin-binding, acid-, heat- and trypsin-sensitive 14–18-kD peptides that crossreact with antisera against aFGF and bFGF. Further evidence includes (a) prevention of mitogenicity by protamine and by anti-bFGF, (b) displacement of ¹²⁵I-bFGF from cell membranes, and (c) stimulation of capillary endothelial cell migration. Specific immunohistochemistry localized bFGF to endothelial cells and, surprisingly, to cardiac myocytes, with almost no immunoreactivity in smooth muscle cells. These peptides may function in cardiac embryogenesis, hypertrophy, atherogenesis, angiogenesis, and wound healing, and may also have endocrine, neurotropic, or vasomotor functions. (*J. Clin. Invest.* 1990; 85:433–441.) fibroblast growth factors • angiogenesis • embryogenesis • atherogenesis • hypertrophy

Introduction

The peptides of the acidic and basic fibroblast growth factor families (aFGF and bFGF, respectively)¹ have recently been purified and sequenced, and their genes have been cloned and expressed (1, 2). While little is known of their endogenous function, their administration in vivo leads to angiogenesis (3–5) and, in the early embryo, bFGF with transforming growth factor-beta 2 leads to induction of the mesoderm (6, 7). Addition of these peptides to cultures of cells of mesodermal or neuro-ectodermal origin, such as fibroblasts and endothelial and smooth muscle cells, causes proliferation (8). Because these peptides are found within cultured cells and their extracellular matrix, but not in plasma, it is presumed that they serve an autocrine or paracrine role in vivo (9–11).

Originally identified in pituitary and brain, bFGF has been found in a number of other tissues (see reference 8) with at

least two exceptions: serum (9) and thyroid (Gospodarowicz, D., personal communication). In culture, bFGF is found in endothelial cells (9–11) and many other normal and transformed cell types, with some exceptions (12–15). aFGF is essentially restricted to brain (5, 16) and retina (17), with small amounts identified in kidney (18) and bone matrix (19). In culture it is expressed by smooth muscle (20) and rhabdomyosarcoma (21) cells, but not by several other cell types (12–14). Little (10) or no aFGF (9, 11, 22) is expressed by cultured capillary, aortic, or umbilical vein endothelial cells. The amounts, sizes, and immunoreactivity of these peptides also differ depending on the species and organ extracted (2, 23, 24). If present in the heart, these peptides could be involved in embryogenesis, angiogenesis, atherogenesis, hypertrophy, or healing of infarction. However, in one early study little or no bFGF was identified in the heart (25). Though there is no established correlation of tissue FGF content with indices of cell proliferation, small amounts of aFGF and bFGF would at least be consistent with the very low rates of cell proliferation in adult heart (26) and with the inability of adult heart tissue to stimulate angiogenesis in the chorioallantoic membrane assay (27). More recently, however, endothelial cell mitogenic activity was extracted from ischemic rabbit heart (28), and aFGF has been identified in cultured neonatal rat cardiac myocytes (29). There are also recent reports from our lab and others of heparin-binding mitogens from postmortem samples of normal human hearts (30) from atrial tissue of patients with heart disease (31), and from bovine, porcine, and canine hearts (32). The present investigation was undertaken to resolve the question of whether cardiac tissue contains either aFGF or bFGF, and if so, how much and in what form, and from which cells.

Methods

Patients. Samples were obtained after examination by the pathologist. Sample 1 was obtained from a 63-yr-old man who died on 2 September 1986 of pneumonia after a 2-yr course of adenocarcinoma of the esophagus, for which he had received radiation therapy and idoxuridine. He had no history of heart disease and the heart was normal on examination 6 h postmortem. A 58-g portion of the left ventricle was frozen at –70°C until extraction on 14 September 1986.

Sample 2 was obtained from a 70-yr-old woman who died on 5 September 1986 of debilitation, secondary to metastatic adenocarcinoma of the breast and recent excision of a pancreatic abscess. She had received radiation therapy and tamoxifen. Her only cardiac history was that of mitral valve prolapse, which had been documented at cardiac catheterization in 1980. Her medications were propranolol, tamoxifen, and morphine sulfate. The interval to postmortem examination was 12 h. The heart was grossly and microscopically normal.

Sample 3 was obtained from a 25-yr-old male victim of a motor vehicle accident who, once it was determined that he was brain dead,

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1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; GFU, growth factor unit; hr, human recombinant; LV, left ventricle; RA, right atrium.

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had several of his organs harvested intraoperatively for potential transplantation, with informed consent by the next of kin, and in accordance with the protocols of the New England Organ Bank and the Committee for the Protection of Human Subjects in Research, Brigham and Women's Hospital, Boston, MA. Although there was no history of heart disease, the heart was not harvested for transplantation because of the presence of pneumonia. In the operating room, 21 g of left ventricle were resected and immediately frozen in liquid nitrogen. Other muscle strips were tested and found to have normal contractility on the day of extraction, 28 January 1987.

Sample 4 was obtained from a 75-yr-old woman with no history of heart disease who died on 7 January 1988 of disseminated ovarian carcinoma. The heart was grossly and microscopically normal. 22 g of left ventricular (LV) apex and 2 g of right atrium (RA) were obtained 2 h postmortem.

Rat and dog myocardium. None of the human tissues could be cannulated and rinsed of blood. This raised the possibility that any mitogenic activity could be due to adsorbed platelet or plasma proteins. For this reason we also extracted blood-free hearts from ten 250-g male Sprague-Dawley rats (Taconic Farms, Inc., Germantown, NY) and from a 22-kg male foxhound and a 12-kg female beagle National Institutes of Health (NIH). These animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publ. No. NIH 80-23) under the supervision of the Section on Laboratory Animal Medicine and Surgery, National Heart, Lung, and Blood Institute (NHLBI), and were free of disease. The protocol was approved by the NHLBI Animal Care and Use Committee. Briefly, the rats were anesthetized with pentobarbital (50 mg/kg i.p.), the chest was opened, and the heart was removed and immediately perfused with saline by an aortic cannula until the perfusate became clear. Normal dog hearts were likewise removed and perfused at the termination of unrelated studies.

Tissue extraction and growth factor purification. Each myocardial sample was analyzed separately. Each was frozen in liquid N₂, pulverized, and mixed with 8 vol of cold 1 M NaCl in 10 mM Tris, pH 4.5 or 7.4, with 1 mM EDTA, 2 mM PMSF, 0.5 mM Na tetrathionate, 1 µg/ml pepstatin A (all from Sigma Chemical Co., St. Louis, MO), plus 1 µg/ml leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN). The tissue was then briefly homogenized by Polytron (Lucerne, Switzerland) and centrifuged at 50,000 g for 60 min at 4°C. The fat was skimmed and the supernatant diluted to 0.5 M NaCl, verified by conductivity testing (Radiometer, Copenhagen, Denmark). It was then adsorbed at 4°C overnight (with gentle rocking) to swollen heparin-Sepharose (1 ml per 10 g of wet tissue; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.5 M NaCl and 10 mM Tris. The nonadsorbed supernatant was decanted and the beads were poured into a column and washed with 20 column vol of 0.5 M NaCl with 10 mM Tris. The column was then eluted with 0.6-ml fractions of NaCl, as either a continuous or stepped gradient of 1.1, 1.5, and 2 M NaCl/10 mM Tris (rate, 20 ml/h) for a total elution volume of 1 ml/g wet weight of heart. The fractions were collected in iced polypropylene tubes and frozen at -80°C.

SDS-PAGE. SDS-PAGE was carried out by a modified Laemmli technique using 10–20% gradient mini-gels (ISS; Hyde Park, MA) and prestained and unstained molecular weight markers (ISS). All samples were adjusted to equal ionic strengths, usually 0.8 M, reduced with beta mercaptoethanol, and heated to 90°C for 3 min. Additional molecular weight markers were human recombinant (hr) bFGF and aFGF (154 amino acid constructs), which usually migrate at 17,500 (a generous gift of Dr. L. Cousens and Dr. P. Barr, Chiron Corp., Emeryville, CA). The gels were run for 2.5 h at 35 mA and 100–150 V as mirror images so that half the gel could then be stained by the silver technique using the ISS kit and the other half of the gel immediately placed in transfer buffer.

Western immunoblotting. The gels were immediately transferred to 0.05-µm pore nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) at 30 V, 200 mA, over 45 min in a Polyblot semi-dry apparatus (American Bionetics, Hayward, CA) using the manufacturer's speci-

fied anode and cathode buffers. The proteins were then stained as described below.

Antibodies. Several polyclonal antisera were raised in rabbits (by Dr. Sasse) using peptide replicates of bovine bFGF (original numbers: 1, 2, 3 = PAL), 1–12, 33–43, and 135–145, synthesized as described (33). In Western blotting these detect 10 ng of hrbFGF and 100 ng hraFGF (10% crossreactivity). Rabbit anti-bFGF_{1–24}, a generous gift of Dr. Andrew Baird (Whittier Institute, La Jolla, CA), detects 5 ng hrbFGF (and 100 ng hraFGF) in Western blotting. Its epitope, residues 11–15, is without homology for predicted products of the oncogenes int-2, hst (K-FGF), and FGF-5.

Dr. Denis Gospodarowicz (Cancer Research Institute, University of California, San Francisco, CA) generously provided an IgG-purified rabbit polyclonal antiserum ("cat," raised against whole denatured hrbFGF) which detects 10 ng hrbFGF in a Western blot at a 1:1,000 dilution. 10 µl blocks the mitogenic effect of 0.4 ng bFGF.

Polyclonal antisera against human aFGF residues 7–16 and 50–82 (numbered as in the original sequence, beginning FNLR . . .) were raised by Dr. Sasse as above. We also used a polyclonal anti-aFGF_{22–155} (a generous gift of Dr. Michael Jaye, and numbered according to his deduced sequence, beginning AEGE . . .). All three detect 5 ng hraFGF in a Western blot with 1–10% crossreactivity with hrbFGF. Anti-aFGF_{22–155} also crossreacted with BSA.

Dot blotting. 10- or 20-µl samples were applied to 0.05-µm pore nitrocellulose sheets clamped in a Minifold II apparatus from Schleicher & Schuell, Inc. The samples were allowed to adsorb for several hours until dry. Nonspecific sites were then blocked by incubating in 5 mg/ml gelatin in PBS for 30 min and then in 10% goat serum in PBS for 60 min. The samples were rinsed with three 1-min washes of PBS with 0.1% Tween and then the primary antiserum was applied and allowed to incubate for 10–12 h. The dilutions were as follows: anti-bFGF_{1–24}, 1:80,000; anti-bFGF_{33–43}, 1:1,000; anti-bFGF cat, 1:4,000; anti-aFGF_{50–82}, 1:20,000; anti-aFGF_{7–16}, 1:10,000; and anti-aFGF_{22–155}, 1:40,000. After three 5-min washes the samples were incubated with biotinylated goat anti-rabbit IgG (Zymed) at 1:1,000 dilution for 1 h followed by three washes (5 min each). They were then incubated in the biotinylated peroxidase-streptavidin complex (Amersham Corp., Arlington Heights, IL) at a dilution of 1:330 for half an hour, followed by application of the substrates that consisted of 16 µl of 30% hydrogen peroxide in 40 ml of PBS with 24 ng of 4-chloro-*l*-naphthol in 8 ml methanol (all from Sigma Chemical Co.). The color intensities were graded by eye using a 1–4+ scale.

Cell culture. Balb/c 3T3 mouse fibroblasts, clone A31 (American Type Culture Collection, Rockville, MD), were grown in 75-cm² plastic flasks (Corning Glass Works, Oneonta, NY) in DME with 10% calf serum and 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin, 0.2 µm-filtered (Nalge Co., Rochester, NY), in a humidified 37°C incubator with 10% CO₂. All reagents were from Biofluids (Rockville, MD). The cells were negative for mycoplasma by Genprobe (Fisher Scientific Co., Pittsburgh, PA). They were split at 70% confluence with 0.05% trypsin-EDTA at a split ratio of 1:1,000. Cells were not used after passage 10.

Bovine adrenal capillary endothelial cells were isolated as described (34) and maintained in gelatin-coated T-75 flasks in DME (low glucose) with 10% calf serum, 1 ng/ml of bFGF every other day, 50 U/ml penicillin, and 50 µg/ml streptomycin, in a humidified 37°C incubator with 5% CO₂. Cells were passaged weekly at a split ratio of 1:64 and studied between passages 6 and 9.

Fibroblast mitogenicity was assayed using the Balb/c 3T3 fibroblasts as described (19).

Capillary endothelial cells were plated at 10,000/2 ml in gelatin-coated 35-mm Falcon dishes (Becton Dickinson, Oxnard, CA) in 10% calf serum, with 0–5 ng/ml bFGF on days 1 and 3 as a standard curve, and 1, 2, or 3 µl of the column fractions (or elution buffer, as control) on days 1 and 3. Cells were trypsinized and counted by electronic particle counter (Elzone) on day 5.

Protein quantification. Protein content was measured by the Bradford method using kits from Bio-Rad Laboratories.

Inactivation studies. Active 1.5 M fractions, and bFGF as positive control, were inactivated by boiling for 10 min, by acidification in 0.1 N HCl followed by neutralization, or by L-1-tosylamide-2-phenylethyl chloromethyl ketone trypsin, 50 $\mu\text{g}/\text{ml}$ at 37°C for 100 min, to which 150 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor was added before (control) or after the incubation.

FGF receptor blockade. Protamine (5 $\mu\text{g}/\text{ml}$), which blocks the FGF and platelet-derived growth factor receptors (35), was used to block the mitogenicity of the 1.5 M fractions, and tested with and without hrbFGF as positive and negative controls.

Anti-bFGF blocking antiserum. 20 μl of a 1:1,000 dilution of Dr. D. Gospodarowicz's antiserum (described above) was added to Balb/c 3T3 cells alone (as background) or just before addition of 0.8 ng hrbFGF (as control) or 0.5 μl of a 2 M heparin column fraction of human heart, to determine whether that fraction's mitogenicity was due to bFGF.

Estimates of FGF equivalents. Acidic FGF-like mitogenicity (that eluting between 0.9 and 1.3 M NaCl by gradient or with 1.1 M stepped elution) was estimated by comparison with a standard curve using recombinant aFGF or bFGF (generous gifts of L. Couzens and P. Barr, Chiron Corp.), or from serum growth factor units (GFUs) using the convention 1 GFU = $\frac{1}{2}$ (maximal 3T3 thymidine incorporation in response to serum minus background). In some assays there was substantial overlap of the two peaks, and immunoblotting (see below) indicated that some bFGF eluted with 1.3 M NaCl, while some aFGF eluted at 1.5 M NaCl. Our assumption that these overlaps cancel out is one of several possible sources of error in quantitation of aFGF and bFGF contents.

Basic FGF-like mitogenicity was assumed to be that eluting between 1.4 and 2 M by gradient, or by 1.5 and 2 M stepped elutions. In these Balb/c 3T3 assays 1 ng/ml of purified pituitary bFGF or 2 ng of brain aFGF (generous gifts of D. Gospodarowicz) or 1 ng/ml hrbFGF or 2 ng/ml hraFGF, equaled 3 GFUs (though depending on the culture conditions bFGF is often 10-fold more potent than aFGF, especially for capillary endothelial cell proliferation).

Radioreceptor assay. BHK-21 fibroblasts were grown as described by Neufeld and Gospodarowicz (36) and assayed in confluent monolayer culture at 4°C. Human rbFGF (Chiron Corp.) was iodinated with iodogen as described, to an estimated sp act of 2×10^5 cpm/ng (36).

After 2 h at 37°C in serum-free DME with 0.15% gelatin, cells were washed, blocked with 2 μg bFGF/16-mm well, and incubated for 2 h at 4°C with ^{125}I -bFGF (0.432 ng/well) plus 0.2–20 ng/well bFGF. Low affinity binding was removed by a 2-min wash with 2 M NaCl and radioactivity was extracted with Triton X-100 as described by Moscatelli (37).

Cell migration assay. Migration was assayed using 10^5 freshly trypsinized, newly confluent capillary endothelial cells, seeded onto polyvinyl pyrrolidone-free filters (Nucleopore Corp., Pleasanton, CA), 6 μm pore size, and coated with 5 μg collagen IV (Collaborative Research Inc., Lexington, MA) in blind well Boyden chambers, essentially as described (38). Cells on the lower surface were counted under the microscope after 4 h of migration at 37°C. No attempt was made to distinguish chemokinetic from chemotactic activity by checkerboard assay.

Immunohistochemistry. Rat hearts were fixed by perfusion with 10% formalin. 6- μm paraffin sections were rehydrated and endogenous peroxidases were quenched by 2% H_2O_2 in methanol for 30 min at 24°C, washed with a surfactant (1:400 BLJ-35; Sigma Chemical Co.), and partially digested with hyaluronidase (2 mg/ml in acetate buffer, pH 5.5, for 15 min at 24°C) (39). Slides were again washed, blocked with 5% goat serum, washed, and incubated with either anti-bFGF₁₋₂₄ or anti-bFGF (cat) at 4°C overnight. Goat anti-rabbit antiserum (1:200, 1 h, 24°C), ABC complex (Vector Laboratories, Burlingame, CA), and diaminobenzidine were used according to the manufacturer's instructions. The counterstain was 1% methyl green. Specificity was indicated by the virtual absence of stain with nonimmune serum, or with immune serum if sections were prewashed with 2 M NaCl to remove bFGF associated with heparan sulfate. Almost no stain was

seen when anti-bFGF₁₋₂₄ was adsorbed with excess hrbFGF but not with hraFGF. Peptide adsorption was not tested with cat, but the stain pattern was similar to that with anti-bFGF₁₋₂₄.

Results

Mitogen assays

Fig. 1 shows the mitogenic effects, for fibroblasts and capillary endothelial cells, of a representative extract (heart No. 2) of human LV myocardium extracted in high ionic strength buffer at pH 4.5. Mitogenic activity for 3T3 fibroblasts and capillary endothelial cells was present in fractions that eluted from heparin-Sepharose columns in broad peaks at 1 and 1.5 M NaCl, consistent with the heparin affinity of aFGF and bFGF, respectively. The other hearts yielded peaks that were less well separated, with the bFGF peak generally larger. No other proliferation studies were performed with the 1 M fractions. However, further evidence of similarity, if not identity, of the 1.5 M column fractions with bFGF is indicated by the loss of activity with boiling, trypsinization, or acid treatment. The antiserum (cat) to whole bFGF and the FGF receptor blocker protamine each reduced the mitogenicity of these fractions by > 90% (data not shown).

To exclude the possibility that the mitogenic activity was not from heart tissue, but from residual plasma or serum, we analyzed platelet-poor plasma, whole blood serum, and fresh uncentrifuged whole blood from three healthy male volunteers, ages 23, 29, and 31 yr (data not shown). There was little or no heparin-binding mitogenic activity in any of these 40-ml samples, which would represent 10 times the amount of blood in a perfused 20-g sample of myocardium. The data suggest

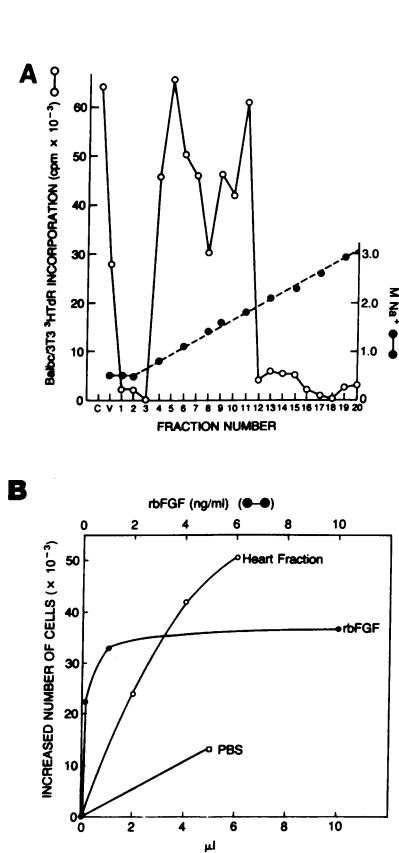


Figure 1. A, Mitogenicity for Balb/c-3T3 cells of crude human heart supernatant (c on x-axis), non-heparin-binding supernatant (v on x-axis), and heparin-Sepharose column fractions. Background counts have been subtracted. Counts of triplicate wells were within 10% of the mean. This experiment was repeated at least once for each heart. **B,** Proliferation of capillary endothelial cells in response to recombinant bFGF (rbFGF), 1.5 M heparin column fractions of heart extract, or PBS control. Initial numbers of attached cells at 4 h (~4,000), when test substances were added, have been subtracted. Counts of triplicate wells were within 15% of the mean. The experiment was repeated once.

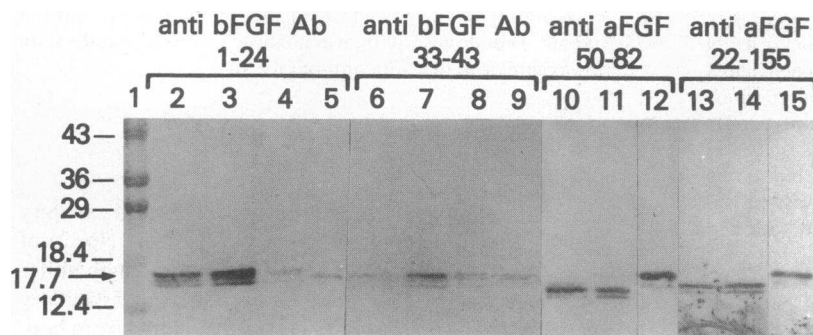


Figure 2. Western blots of fractions of human heart No. 3 eluted from heparin-Sepharose with 1.5 M NaCl and incubated with anti-bFGF₁₋₂₄ and anti-bFGF₃₃₋₄₃, and of 1.1-M fractions incubated with anti-aFGF₅₀₋₈₂ or anti-aFGF₂₂₋₁₅₅. Lanes 5 and 9 are recombinant bFGF. Lanes 12 and 15 are recombinant bFGF. Lanes 2-4, 6, and 7 are 1.5-M fractions. Lanes 10, 11, 13, and 14 are 1.1-M fractions. PreadSORption of anti-bFGF resulted in no stain (not shown). Similar results were obtained with other hearts assayed by these antisera and by anti-bFGF₁₃₅₋₁₄₅, anti-hrbFGF (cat), and anti-aFGF₇₋₁₆. However, no aFGF immunoreactivity was found in heart No. 4.

that there could be no more than 10 ng of aFGF or bFGF per ml of blood. We also identified these heparin-binding immunoreactive mitogens from rat and canine hearts after perfusing them until free of blood (data not shown).

Western blotting

1.5 M NaCl eluate. The heart-derived mitogen that eluted at 1.5 M NaCl reacted with antibodies raised against bFGF residues 1-24 and 33-43 (Fig. 2). Not shown are Western blots demonstrating reactivity with the antisera to hrbFGF and to the COOH terminus, as well as a dot blot showing reactivity with a polyclonal antiserum raised against amino acids 1-12. No reaction product was detected in the absence of immune serum, or if the immune serum was combined with excess hrbFGF (not shown).

Bands were seen only at M_r 14 kD in the first heart, but in subsequent hearts bands were noted at 18, 17, and 16 kD, consistent with the well-recognized NH₂-terminal cleavage of bFGF by acid proteases. We also noted that 14-kD bands retained some NH₂-terminal immunoreactivity, suggesting that cleavage can also occur at the COOH terminus. After a few days to weeks at 4°C, impure fractions often revealed only 14-16-kD bands, suggesting that a protease can copurify with the mitogen (Fig. 3).

The detection of 18-kD immunoreactivity, migrating more slowly than the 154 residue hrbFGF that corresponds to the published gene sequence, is consistent with other recent reports of extended forms of bFGF (23).

1 M NaCl eluate. The mitogen eluting at 1 M exhibited

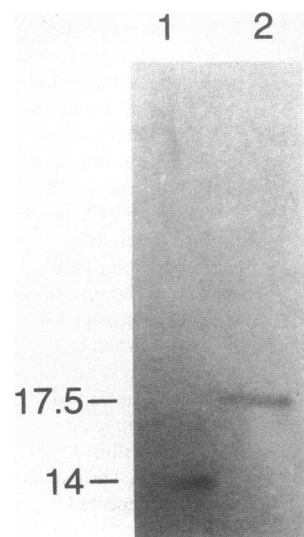


Figure 3. SDS polyacrylamide gel, stained with silver, of mitogen eluted by 1.5 M NaCl from heparin-Sepharose column and, several weeks later, separated from high molecular weight contaminants by gel filtration, by which time the mitogen (lane 1, now purified to single band homogeneity), which formerly migrated with the recombinant bFGF (lane 2), had apparently been cleaved to M_r 14 kD. Unmarked lane, molecular weight markers $\times 10^{-3}$.

immunoreactivity with antibodies raised to residues 7-16 and 50-82 of bovine aFGF and to a polyclonal antibody raised against whole human aFGF, as shown in Fig. 2. Gel electrophoresis usually indicated a mitogen of $\sim 15,000 M_r$, but immunoreactivity was occasionally noted at 17 kD as well. Some bFGF immunoreactivity was usually seen also, especially in the later 1 M fractions.

Purification and quantification of aFGF and bFGF

After one step of heparin-Sepharose chromatography silver-stained SDS polyacrylamide gels usually revealed 6-20 bands in the 1.1 M fractions, 3-10 bands in the 1.5 M fractions, and 2-3 bands in the 2 M fractions. Subsequent purification to single band homogeneity (Fig. 3) was achieved by gel filtration, or cation-exchange chromatography, or in one case by two more steps of heparin-Sepharose chromatography. In heart No. 1, single band purity was achieved after only one step of heparin-Sepharose chromatography.

The content of aFGF and bFGF were estimated after one cycle of heparin-Sepharose chromatography, based on GFU, substantiated by estimates of immunoreactivity based on slot blots using recombinant peptides as standard curves (density of reaction product correlated with amounts of hrbFGF or hraFGF from 100 pg to 25 ng). Estimates for LV samples are shown in Table I. The right atrial sample from patient 4 yielded only 30 ng/g. However, for unknown reasons there was only 10% as much protein in the supernatant of the atrium compared with that of the ventricle, so that the atrium and ventricle had equal mitogenicity when expressed as bFGF equivalents per milligram of supernatant protein. No other human samples were available to repeat the experiment. However, bovine RA and LV yielded, per gram of wet tissue, similar amounts of bFGF (calculated from serum GFUs), 380 ng (RA) vs. 400 ng (LV) per gram, and similar amounts of protein in the supernatant of the crude homogenate, 26.6 mg (RA) vs. 24.6 mg (LV). However, the same heart yielded more aFGF in the LV (200 ng/g) than in the RA (100 ng/g).

Table I. Amounts of aFGF and bFGF (nanograms/gram tissue) from LV Samples of Four Human Hearts

	Sample No.			
	1	2	3	4
aFGF (ng/g)	250	260	454	0
bFGF (ng/g)	218	130	388	295

Amounts are estimated from Balb/c 3T3 mitogen assays as described in Methods.

As a preliminary attempt to detect organ-specific differences, whole brain and whole heart tissue from the same dog were homogenized in 1 M NaCl, pH 4.5, and subjected to heparin-affinity chromatography in the usual fashion. These extractions yielded the following: protein, 5 mg/g (brain), 6.2 mg/g (heart); aFGF, 0.2 μ g/g (brain), 0.36 μ g/g (heart); bFGF 2 μ g/g (brain), 4.2 μ g/g (heart).

The variation between patients could reflect interindividual differences based on age, sex, clinical condition, or other factors. However, there were also differences in the time intervals between death and tissue harvesting, and time in the freezer, as well as some differences in purification procedures. For example, we have noted that recovery is influenced by the volume, pH, and ionic strength of the homogenization buffer, the speed of centrifugation, and the freshness of heparin-Sepharose and its lot-to-lot variability. The degree of purification obtained with one cycle of heparin-Sepharose chromatography varies from \sim 1,000- to 6,000-fold. Thus, even with simultaneous mitogen and immunoassays it is difficult to determine whether there are interindividual or interspecies variations based on so few samples.

Estimates of recovery are likewise problematic. The supernatant of the crude homogenate (the conventional denominator) necessarily excludes any growth activity in the pellet. Secondly, the heart or the homogenization buffer may contain inhibitors of 3T3 cell growth, causing the content of FGF peptides in the supernatant to be underestimated and their recovery overestimated. The presence of mitogens other than FGFs in the heart would lead to the opposite error.

Radioreceptor assay

The 1.5 M column fractions displaced 125 I-bFGF from monolayers of BHK-21 cells in a manner similar to hrbFGF (Fig. 4). The 1.1 M fractions were not tested.

Cell migration assay

The 1.5 M column fractions duplicated the effects of hrbFGF in promoting migration of capillary endothelial cells (Fig. 5).

Immunohistochemistry

Specific staining for immunoreactive bFGF was prominent in rat ventricular cardiac myocytes and arterial endothelium (Fig.

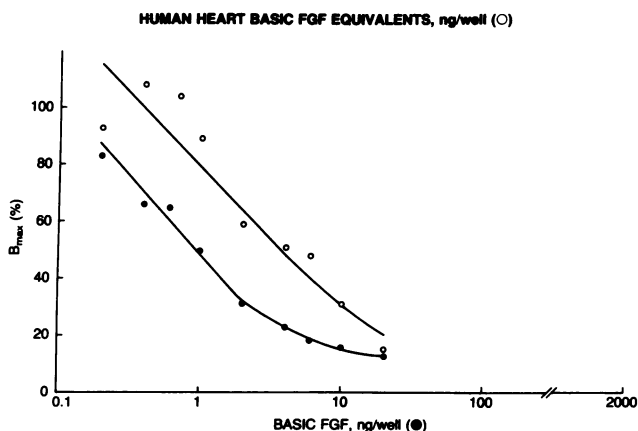


Figure 4. Radioreceptor assay for bFGF. 125 I-bFGF bound to BHK-21 cell monolayers was displaced by hrbFGF and heart-derived bFGF as described in Methods. Nonspecific binding (6%) was subtracted from values shown. Counts of triplicate wells were within 15% of the means. Assay was repeated once.

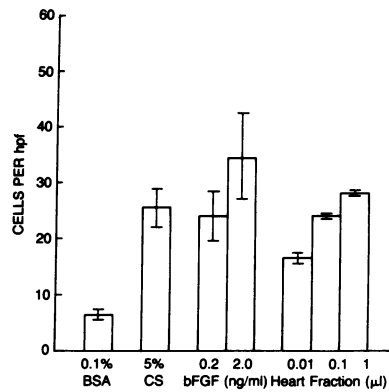


Figure 5. Boyden chamber assay of migration of capillary endothelial cells to BSA, recombinant bFGF, 5% calf serum (CS), or aliquots of 1.5 M heparin column fractions of human heart. Values are means \pm SD of duplicate filters. The experiment was repeated once.

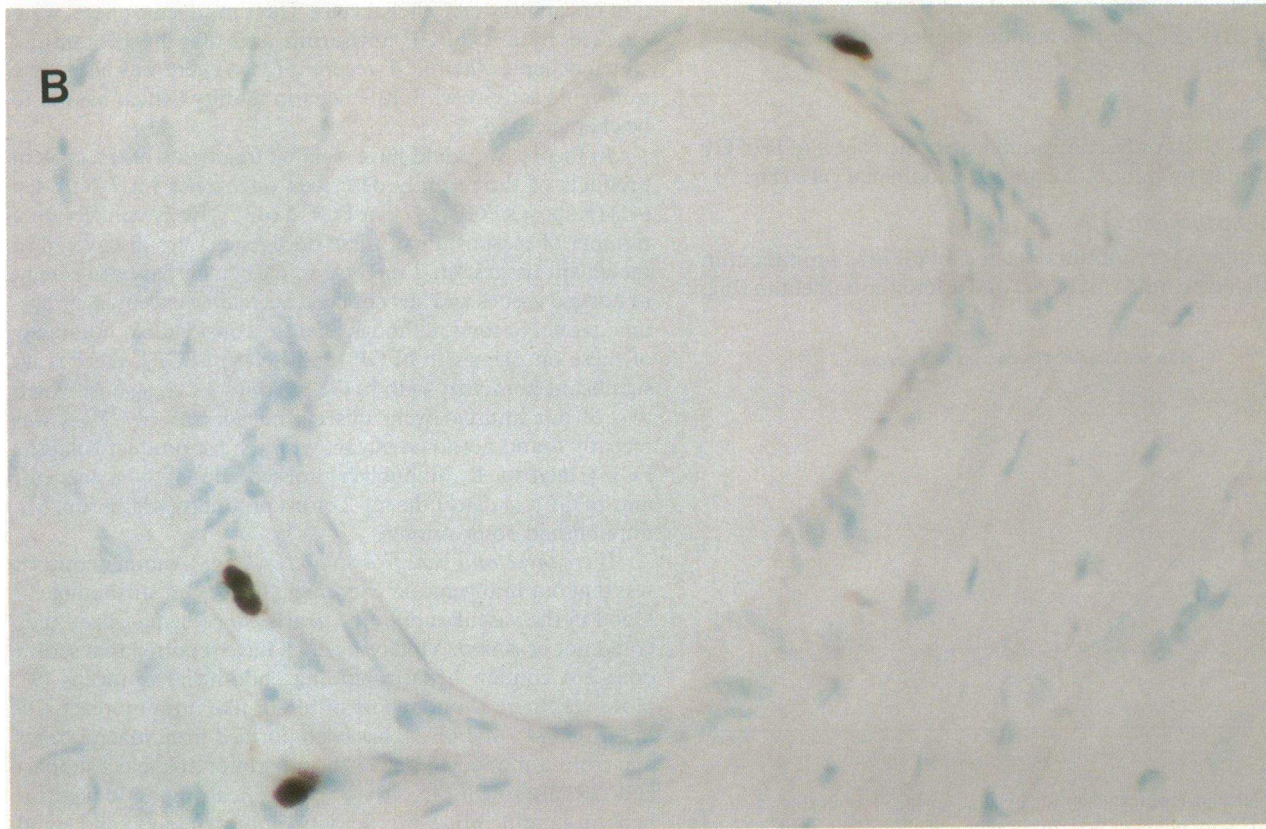
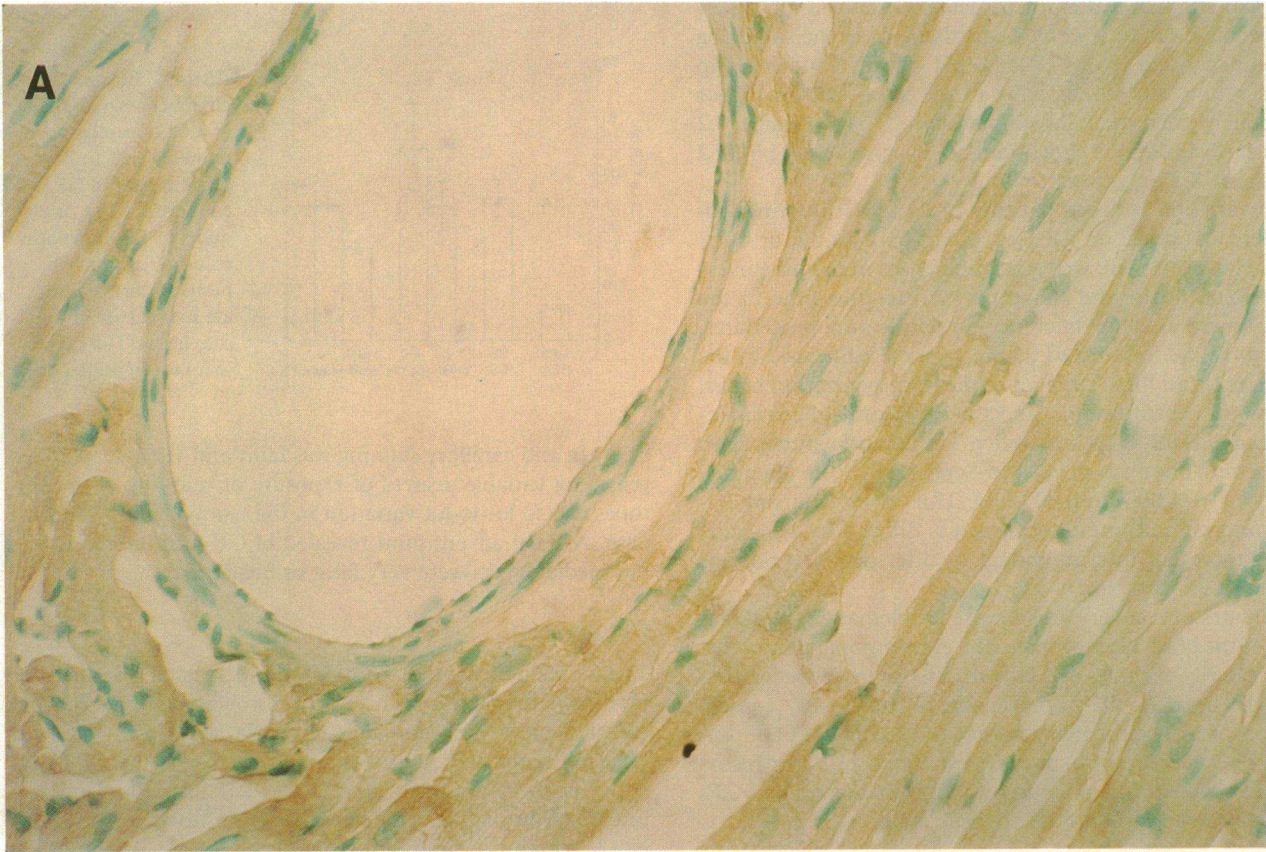
6). Vein and capillary staining was faint and variable, perhaps reflecting variable degrees of exposure or masking of the epitopes due to lot-to-lot variation in the fixative and hyaluronidase. Arterial adventitium revealed bFGF immunoreactivity, but medial layers were very faint or unstained.

Discussion

These data indicate that normal human atrial and ventricular myocardium contain mitogens for fibroblasts and endothelial cells and that these mitogens resemble aFGF and bFGF in their molecular weight and affinity for heparin, their sensitivity to heat, acid, and trypsin, and their immunoreactivity. Fractions eluting from the heparin column between 1.2–2 M NaCl, the elution profile for bFGF, competed with radiolabeled bFGF for cell membrane binding and promoted migration of capillary endothelial cells. Their mitogenic effects were blocked by anti-bFGF antiserum and also by protamine, which occupies the bFGF receptor (35). There was also some mitogenic activity with little heparin affinity, which has yet to be characterized.

In theory, we could have isolated fragments of the protein products of the recently described oncogenes int-2 (40), hst (41) (Kaposi's FGF) (42), or FGF-5 (43). This is unlikely for a number of reasons. First, these oncogenes were discovered in malignant tissues; int-2 mRNA, in particular, has been sought in normal tissues and has not been detectable except in embryonic tissue. Second, although there is considerable homology of these oncogenes to bFGF (and less to aFGF), there is no significant homology with bFGF residues 1–24, against which two of our antisera were raised. Indeed, anti-bFGF_{1–24} was recently found not to crossreact with the hst product Kaposi's FGF (Halaban, R., unpublished observations). Nor does our anti-bFGF_{33–43} detect the int-2 or hst products (Klagsbrun, M., unpublished observations).

Were these mitogens from blood or heart? Another concern was that our mitogenic activity might have been attributable to blood in the vasculature of the hearts, since being frozen they could not be rinsed. Vlodaysky et al. have reported that serum does not contain heparin-binding endothelial mitogens (9). However, there is one report of bFGF-like immunoreactivity in serum (44), and bFGF has been purified from macrophages and from a monocyte line (24). In addition, platelets support endothelial growth (45, 46). For these reasons we looked for heparin-binding mitogenic activity in human blood and could not find any. Furthermore, blood-free dog and rat hearts yielded heparin-binding mitogens with similar immunoreactivity. Finally, the immunohistochemical staining makes it



very likely that the activity we have detected derives from the myocardium and not from blood.

Are these mitogens an artifact of agonal synthesis? We studied normal tissue to exclude the possibility that any FGF-like activity was due to such processes as atherosclerosis, hypertrophy, or malignancy. Galloway et al. (28) had found endothelial mitogenic activity in ischemic rabbit heart, and we had previously isolated heparin-binding mitogens from ischemic canine (47) and postmortem human myocardium (30). These findings, plus earlier reports of the lack of mitogenic activity in crude heart extracts (25) or of angiogenic activity of adult heart tissue grafts (27) in the chorio-allantoic membrane, together with the rarity of cell division in the adult heart (26), suggested the possibility that bFGF and aFGF might be absent in normal heart, but rapidly synthesized in response to ischemia. The well-established decline in overall protein synthesis in ischemic myocardium (48) did not preclude such a possibility, since there is some evidence of new synthetic activity in the acutely stressed heart (49). Stress protein synthesis in response to ischemia (50) and platelet-derived growth factor synthesis by dying cells (51) are well-documented responses that suggest that specific FGF synthesis could occur as an agonal event. Hence, we made certain to obtain a sample of absolutely fresh, nonischemic myocardium, and this sample (No. 3) did contain both aFGF and bFGF.

Amounts of myocardial FGF peptides. Differences in age, sex, and clinical conditions preclude any precise comparison of the amounts in the four samples. Nor can exact comparisons be made with FGF yields from other organs, since we were unable to obtain comparably fresh brain or pituitary tissue from the same patients. We did compare brain and heart from the same dog, in parallel, and found that the heart contained more aFGF and bFGF than brain.

We were also surprised to find in most hearts somewhat more aFGF than bFGF. Similar findings have been reported for brain tissue. (The apparent absence of aFGF in one heart may be an artifact or may reflect the greater susceptibility of aFGF to proteolysis). We considered the possibility that this finding was a reflection of the particular extraction protocol, but roughly the same results were obtained despite varying pH, ionic strength, and other homogenization conditions (Spear, E., unpublished observations).

Sizes of myocardial FGF-like peptides. The detection of a slightly longer form of bFGF in the heart compared with that found in most tissues has some recent precedence in the detection of a 25-kD form of bFGF, extended at the amino terminus, in guinea pig brain (23).

It also remains unclear whether different sizes of FGF represent alternative sites of initiation of transcription or translation, differential splicing, variable polyadenylation, or processing in vivo or during the extraction procedure. We have purified shorter forms of both aFGF and bFGF, migrating at 14, 15, and 16 kD, from canine, human, and rat myocardium, especially when protease inhibitors were omitted from the homogenization buffer, and have also observed in the human and rat column fractions that the 17- and 18-kD forms of

bFGF can be cleaved to 14 kD over the course of a few weeks at 4°C even when nearly homogeneous by silver stain. This suggests that small amounts of a heparin-binding protease, from serum or myocardium, such as plasmin, trypsin, thrombin, or a cathepsin, may copurify with bFGF on heparin affinity columns. The consistently greater tendency of aFGF, compared with bFGF, to elute as a mitogen of M_r 14 kD, may reflect either the greater sensitivity of aFGF to degradation by thrombin (52) or its lesser affinity for heparin, which protects both mitogens against trypsin (53).

Cellular localization of bFGF. With the exception of the corneal matrix, the localization of bFGF in vivo is not known. In this study we found prominent bFGF immunoreactivity in the arterial endothelium with faint stain in capillary and venular endothelium. The staining was more evident in some cells than others, reflecting either methodological artifact or capillary heterogeneity. Regions where the endothelium had become separated from the vessel, either by enzyme digestion or microtomy, suggested that bFGF was in the subendothelial matrix as well as in the endothelial cells, as previously shown in vitro (10, 11, 22). The resolution of light microscopy has not allowed us to determine whether there is immunoreactive bFGF at the luminal cell membrane. This will be an important issue for resolution by immunoelectron microscopy, since in vitro studies suggest that low affinity binding to heparan sulfate on the cell surface serves as a reservoir for basic FGF (37).

The absence of stain in the arterial smooth muscle layer would not have been predicted from studies of cultured smooth muscle cells (20). However, the result is consistent with our recent findings that freshly isolated rat aortic smooth muscle cells contain very little of these peptides compared with aliquots of the same cells in culture. Adventitial fibroblasts did exhibit bFGF immunoreactivity.

Cardiac myocytes also were immunoreactive for bFGF. The pattern was punctate but homogeneous throughout the cytoplasm. A few nuclei were stained. The presence of nuclear irbFGF in other cells may be cell specific or cell cycle specific (54).

Immunostaining for aFGF has not shown adequate sensitivity and specificity to date.

Possible functions of cardiac FGF peptides. The presence of cardiac FGF peptides naturally raises the question of their function. It is easy to imagine that these proteins might function in cardiac development, atherogenesis, the healing of myocardial infarction, the development of coronary collaterals in response to ischemia, or the development of myocyte hypertrophy or vascular smooth muscle proliferation in hypertension or hypertrophic cardiomyopathy. However, these conditions are rare or nonexistent in rats and dogs and yet we have found these peptides in these species as well. It may be that in the normal adult heart, as in the pituitary, bFGF serves a nonmitogenic endocrine (55) or neurotropic (56) function. There is also one report of vasoconstrictive activity by bFGF in helical strips of rat aorta (57). Alternatively, in light of bFGF's induction of the mesoderm (6, 7), its presence could simply be

Figure 6. Immunohistochemical staining with anti-bFGF₁₋₂₄ in normal 250-g rat LV. *A.* The brown reaction product, not seen with preimmune or peptide-absorbed antisera, or in sections prewashed with 2 M NaCl, indicates presence of bFGF in cardiac myocytes and arterial endothelium. Faint staining is observed in capillary and venular endothelium in some assays, perhaps reflecting lot-to-lot variation in fixative and hyaluronidase. (×1,500). *B.* The virtual absence of immunoreactivity when 2 μg of hrbFGF was preadsorbed to 1 ml of a 1:1,000 dilution of the primary antiserum (×1,500). Three mast cells stain nonspecifically with the immunoperoxidase system because of their biotin content.

a vestige of embryogenesis, or could serve to maintain some differentiated feature. For example, Gospodarowicz has shown that it maintains the monolayer property of cultured endothelial cells (8). It also increases their lifespan fourfold, to as many as 100 population doublings.

Despite the presence of large amounts of cardiac aFGF and bFGF, which not only enhance proliferation in vitro of endothelial and smooth muscle cells and fibroblasts, but also stimulate DNA synthesis in cultured cardiac myocytes (58), there is little vascular and no myocyte proliferation in the normal adult heart (26). Absence of FGF receptors is a possible explanation. We have found specific cardiac binding in vivo of aFGF (59). Although this binding was not competed by heparin, high affinity binding sites in normal heart tissue have not yet been demonstrated. Inhibition of endothelial proliferation may be due to transforming growth factor-beta (60) which we have identified in the heart (39). Heparin (61), laminin (62, 63), or other growth inhibitors may contribute to the terminal differentiation of cardiac myocytes in the presence of potent growth factors.

Future studies can be expected to clarify the roles of the FGF peptides in the heart. Such data could suggest potential therapeutic possibilities. For example, angiogenesis, wound healing, and regeneration are slow or incomplete in some settings, but can be accelerated by FGF peptides, or by heparin, with which they interact (8, 56, 61, 64). FGF-based therapies might be used to promote coronary collateral growth, to speed healing and prevent expansion and rupture in myocardial infarction, and conceivably, to promote myocyte regeneration.

Note added in proof. Immunoreactivity for bFGF has very recently been reported in nuclei and intercalated discs of cardiac myocytes in cryostat sections (Kardami, E., and R. R. Fandrich. 1989. Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. *J. Cell Biol.* 109:1865-1875). We find both FGFs in nuclei in cryostat sections and in our formalin-fixed cultured myocytes (Speir, E., V. Tanner, S. Shrivastav, and W. Casscells, manuscript submitted for publication). Furthermore, bFGF and aFGF can both be purified from nuclei of cultured endothelial cells (Yu, Z.-X., S. Biro, V. Ferrans, and W. Casscells, manuscript submitted for publication). Kardami et al. could not find evidence of aFGF or TGF- β in adult heart. The reasons for these discrepancies with our results are unclear.

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