

Basic Fibroblast Growth Factor in Atria and Ventricles of the Vertebrate Heart

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Abstract. Extracts from atrial and ventricular heart tissue of several species (chicken, rat, sheep, and cow) are strongly mitogenic for chicken skeletal myoblasts, with the highest apparent concentration of biological activity in the atrial extracts. Using several approaches (biological activity assay and biochemical and immunological analyses), we have established that (a) all cardiac extracts contain an 18,000-D peptide which is identified as basic fibroblast growth factor (bFGF) since it elutes from heparin-Sepharose columns at salt concentrations >1.4 M and is recognized by bFGF-specific affinity-purified antibodies; (b) bFGF is more abundant in the atrial extracts in all species so examined; (c) avian cardiac tissue extracts contain the highest concentration of immunoreactive bFGF; and (d) avian ventricles contain a higher relative molecular

mass (23,000-D) bFGF-like peptide which is absent from atrial extracts. Examination of frozen bovine cardiac tissue sections by indirect immunofluorescence using anti-bFGF antibodies shows bFGF-like reactivity associated with nuclei and intercalated discs of muscle fibers. There is substantial accumulation of bFGF around atrial but not ventricular myofibers, resulting most likely from more extensive endomysium in the atria. Blood vessels and single, nonmuscle, connective tissue cells react strongly with the anti-bFGF antibodies. Higher bFGF content and pericellular distribution in atrial muscles suggest a correlation with increased regenerative potential in this tissue. Distribution within the myofibers is intriguing, raising the possibility for an intimate and continuous involvement of bFGF-like components with normal myocardial function.

TISSUES with extensive vascularization accumulate substantial amounts of heparin-binding growth factors (5, 18–20, 25). The best studied members of this category of growth factors are acidic and basic fibroblast growth factors (aFGF and bFGF, respectively)¹ which have 55% sequence homology (13, 15, 20) and a similar spectrum of biological activity, with bFGF being 30 times more potent than aFGF (18–20). bFGF is a multifunctional peptide. It is a powerful mitogen for cells of mesodermal and neuroectodermal origin (18–20), a mesoderm inducer (morphogen) in embryonic development (28, 41), and a differentiation inducer for some cell types (1). Because of its high affinity for heparin (17), bFGF is accumulated in heparan-sulfate-rich extracellular matrices; it binds to retina basement membranes (21) and is extracted from the extracellular matrix of endothelial cells in culture (4). Endothelial cells synthesize bFGF *in vitro* (45) and may be responsible for the high bFGF accumulation in heavily vascularized tissues. Nevertheless, its precise localization in tissues, cellular origin, or local function are still not established. It is believed that bFGF is involved in angiogenesis, wound repair, and regeneration (18–20).

1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; TGF β , transforming growth factor β .

In one type of striated muscle, skeletal muscle, bFGF has a dual effect; it stimulates embryonic myoblast proliferation (25, 26) and inhibits differentiation independently of cell division (42). bFGF is highly mitogenic for adult skeletal muscle stem (satellite) cells (3), is accumulated in satellite cell-rich muscles (25), and is thus considered to participate in muscle regeneration after injury. Regenerating skeletal muscle has high levels of mitogenic activity (7). Interestingly, physiologically occurring components such as heparin-like substances and transforming growth factor β (TGF β) can inhibit the action of bFGF on skeletal muscle (14), presenting a plausible model for regulation *in vivo*.

Another striated muscle, cardiac ventricular myocardium, is considered essentially incapable of regeneration (47). After birth, ventricular myocytes rapidly lose their proliferative potential and become terminally differentiated (47); a sluggish attempt for resumption of DNA synthesis by these cells after tissue damage has been observed (37). Furthermore, there are reports that ventricular myocytes in culture can be induced to resume DNA synthesis (10); this implies environmentally imposed restrictions rather than irreversible loss of this property. Adult atrial myocytes retain their ability for synthesizing DNA *in vivo* to a considerable extent (38). Little is known about the molecular mechanisms governing proliferation of cardiac myocytes. As with skeletal myoblasts

and satellite cells, bFGF is one of the factors recently shown to stimulate DNA synthesis of adult ventricular myocytes (9). We have also found bFGF to stimulate proliferation of both atrial and ventricular myocytes from neonates, an activity inhibited by TGF β (21, 22). These similarities between the two muscle types led us to hypothesize that, as with skeletal muscle (24, 25), activities present in the cardiac milieu could affect cardiomyocyte proliferation and differentiation and possibly account for differences between adult atrial and ventricular myocytes. To investigate this, we have examined the biological activity of heart extracts and quantitated bFGF-like peptides in atria and ventricles of avian (chicken) and mammalian (rat, sheep, and cow) species; in addition we studied the localization of bFGF-like reactivities in the heart. We found differences in quantity and distribution of bFGF-like components between atria and ventricles which could have a bearing on their respective proliferative properties. Furthermore, the observed intimate association with cardiac myofibers raises the possibility for a continuous involvement of bFGF-like peptides with normal cardiac myocyte properties.

Materials and Methods

Cell Culture

Skeletal myoblasts from 12-d chick embryos, obtained as previously described (26), were cultured on gelatin (0.3%)–coated dishes in 10% (vol/vol) horse serum (HyClone Laboratories, Logan, UT), 25 μ g/ml chicken transferrin (Cooper Biomedical, Inc., Malvern, PA) in MEM (Gibco Laboratories, Grand Island, NY). Plating density was 15,000 and 50,000 cells per well (96- and 24-well plates, respectively).

Rabbit fetal chondrocytes were a gift from Dr. Friesen (University of Manitoba, Winnipeg, Manitoba, Canada) and were cultured as described (27, 44). For [3 H]thymidine incorporation assays, chondrocytes were plated in 96-well dishes at 5,000 cells/well in 10% FCS (Flow Laboratories, Mississauga, Ontario, Canada) and Ham's F-10 medium (Gibco Laboratories). 24 h after plating, medium was removed and replaced with Ham's F-10 medium (Gibco Laboratories) containing 1% BSA. Extracts, growth factors, and column eluates were subsequently added, followed by 1 μ Ci/well of [3 H]thymidine (Amersham Corp., Arlington Heights, IL). [3 H]thymidine incorporation was determined 20 h later as described (16). Recombinant bovine bFGF was purchased from Amersham Corp. Porcine or human TGF β and TGF β -neutralizing rabbit IgGs were purchased from R & D Systems, Inc. (Minneapolis, MN), and insulin-like growth factor (IGF) I was from Calbiochem-Behring Corp. (San Diego, CA). All cells were maintained at 37°C in a humidified atmosphere of 5% CO $_2$ in air.

Biological Activity of the Extracts

Extracts to be tested for mitogenic activity were added to the cells once, at the time of plating, at a final concentration of 0.1–1 mg/ml. Cells were grown on 96-well plates. [3 H]Thymidine was added to the cells after 20–24 h in vitro at 1 μ Ci/well. [3 H]Thymidine incorporation was determined 10–12 h later (16). To test for differentiation-inhibiting activity, extracts were added to myoblast cultures (in 24-well dishes) at a final concentration of 0.5 mg/ml (in quadruplicate wells). To assess differentiation, cultures were fixed in cold ethanol/formalin (9:1) and stained with eosin and hematoxylin (Sigma Chemical Co., St. Louis, MO) before determining fusion index (nuclei inside myotubes/total number of nuclei); for the latter, four representative fields from each well were photographed and the nuclei inside and outside myotubes were scored (minimum of 500 nuclei per well).

Preparation of Tissue Extracts

All procedures were performed at 4°C unless otherwise specified. Immediately after dissection, heart tissue was cleared of fat and large vessels and either processed immediately or frozen in liquid nitrogen and stored at –70°C. For extraction, tissue was weighed, minced with scissors, and

homogenized briefly in 2 vol (over weight) of MEM with a homogenizer (Polytron; Brinkmann Instruments Co., Rexdale, Ontario, Canada) at low setting. Residual tissue was removed by centrifugation in a centrifuge (Ultracentrifuge; Beckman Instruments, Inc., Fullerton, CA) at 35,000 rpm for 40 min at 4°C, and the supernatant was stored in sterile tubes at –70°C after filtration through low protein-binding 0.2- μ m filters (Millipore-Continental Water Systems, Bedford, MA). Protein concentration was determined using a colorimetric assay (Bradford reagent; Bio-Rad Laboratories, Richmond, CA).

SDS-PAGE

The presence of bFGF in samples was assessed by standard SDS-PAGE (30) in 1.5-mm-thick, 15% polyacrylamide slab gels. Protein molecular mass standards (10,000–100,000 D; Bio-Rad Laboratories) as well as pure recombinant bovine bFGF were included in each analysis. Silver staining of gels was performed as described (31).

Immunoblotting and Autoradiography

Gels were transferred onto nitrocellulose membrane (0.2 μ m; Schleicher & Schuell, Inc., Keene, NH) by electrophoretic transfer (Transblot; Bio-Rad Laboratories). After transfer and blocking of the nonspecific protein-binding sites with 1% BSA-PBS, the nitrocellulose membrane was incubated with 40 ng/ml of the purified anti-bFGF IgGs in wash buffer (10 mM Tris-HCl, pH 8, 0.15 M NaCl, 0.05% Tween-20) overnight at 4°C. Antigen-antibody complexes were visualized by incubating the membrane (8) with 0.1 μ Ci/ml of [125 I]–protein A (Amersham Corp.) in wash buffer for 1 h. Autoradiography was performed with X-omat film (Eastman Kodak Co., Rochester, NY) and intensifying screens at –70°C for 1–7 d. Autoradiograms were scanned with a laser densitometer (Ultrascan 2202; LKB Instruments, Inc., Gaithersburg, MD) to determine the relative intensity of the bands.

Heparin-Sepharose Affinity Chromatography

Heparin-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography was used for bFGF isolation from cardiac extracts. Extracts were made up to 0.6 M NaCl by adding solid NaCl and passed twice through heparin-Sepharose columns equilibrated in 0.6 M NaCl, 10 mM Tris-HCl, pH 7 (binding buffer). For every 10 ml of crude extract (protein concentration 15–17 mg/ml), 1 ml of packed heparin-Sepharose was used. After extensive washing, affinity columns were eluted stepwise with 1.1 and 2.5 M NaCl in 10 mM Tris-HCl, pH 7. The 2.5 M salt eluate was immediately concentrated down to 50–100 μ l by centrifugation using microconcentrators (Centricon-10; Amicon Corp., Danvers, MA). Aliquots from these concentrates were used for protein determinations and immunoblotting.

To quantitate the amount of bFGF present in tissue extracts more accurately, minimizing losses, another approach was used: 1–2 ml of extract was mixed with 50 μ l of settled heparin-Sepharose beads in 0.6 M salt. Suspensions were vortexed softly several times over a period of 2 h at room temperature; heparin-Sepharose beads were allowed to settle, and the supernatant was removed carefully. After several washes with binding buffer, the beads were incubated for an additional 10 min with 1 ml of 1.1 M NaCl, and the resulting supernatant was removed. This was followed by two washes with 0.1 M NaCl in 10 mM Tris-HCl, pH 6.8. Packed beads were finally incubated with 150 μ l of gel sample buffer (0.1 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 5% [vol/vol] beta-mercaptoethanol) and boiled for 5 min. The whole suspension was loaded into one gel lane. Recombinant bFGF, treated similarly, was recovered fully from the heparin beads in the gel and migrated identically to untreated bFGF.

Quantitation of bFGF in Cardiac Extracts

Autoradiograms from the Western blots were scanned by densitometry. Recombinant bFGF was included in all the runs (1–20 ng/lane) and was used for the construction of standard curves, which were linear within the 1–10 ng/lane range. An estimate of the bFGF content of each sample was obtained, provided its scan fell within the standard curve.

Immunological Reagents

Rabbit antiserum against the [1–24]NH $_2$ -terminal peptide from bovine brain bFGF (146 amino acids) was prepared as follows: 2 mg of the peptide (Bachem, Inc., Torrance, CA) was conjugated to 0.5 mg of keyhole limpet hemocyanin with 1-ethyl-3-(3'-dimethylamino-propyl) carbodiimide-HCl

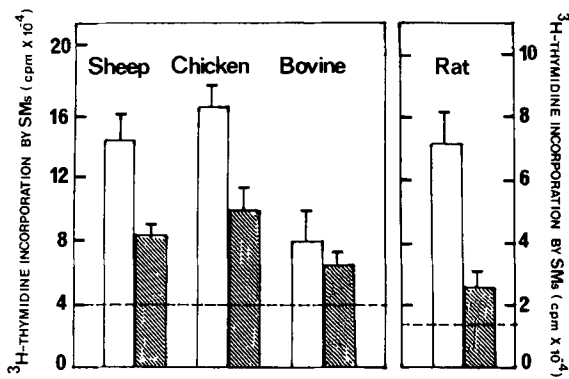


Figure 1. Stimulation of DNA synthesis by cardiac extracts (chicken, rat, sheep, and bovine) in chicken skeletal myoblasts (SMs) after 24 h in culture. Shaded and unshaded columns represent ventricular and atrial extracts, respectively. The broken line shows the level of thymidine incorporation in control cultures (no extract added). Values shown represent the mean of six determinations with the standard deviation indicated as a vertical line.

(Calbiochem-Behring Corp.) as described (40). Two thirds of the resulting conjugate was mixed with Freund's complete adjuvant and used to immunize three rabbits by intradermal injections; the remaining third was mixed with incomplete adjuvant and used for booster injections 3 and 6 wk after the first. Rabbits were killed and immune sera collected 7 wk after the first injection. Purified anti-[1-24]bFGF IgGs were obtained by affinity chromatography: 4 mg of the peptide was conjugated to CNBr-activated Sepharose-4B (1.5 ml bed volume) according to the manufacturer's instructions (Pharmacia Fine Chemicals). Immune serum (1 ml), diluted with 10 ml of 0.5 M NaCl in PBS, was repeatedly absorbed to the column. Specifically bound IgGs were eluted with 4 M of MgCl₂, dialyzed in PBS, and stored at -70°C in the presence of 10% (vol/vol) glycerol. Fluorescein-conjugated (FITC) donkey anti-rabbit IgGs were purchased from Amersham Corp., and rhodamine X-conjugated (XRITC) sheep anti-mouse IgGs were from Cooper Biomedical, Inc. (Malvern PA). Purified monoclonal mouse IgGs, recognizing striated muscle myosin, were a generous gift from Dr. R. Zak (University of Illinois, Chicago, IL).

Immunofluorescence Microscopy

Cardiac tissue was frozen in a dry ice/ethanol bath and used immediately for cryosectioning. Longitudinal or transverse sections 7 μm thick were routinely obtained using a cryostat (1720; E. Leitz, Inc., Wetzlar, FRG). Sections were collected on gelatin-coated slides. They were incubated with the anti-[1-24]bFGF (1-5 μg/ml) with or without antimyosin IgGs (at 2 μg/ml) in 1% (wt/vol) BSA-PBS overnight at 4°C. After washing the sections with cold PBS, they were incubated with fluorescein-conjugated anti-rabbit IgGs and rhodamine X-conjugated anti-mouse IgGs at 1:20 to 1:30 dilutions for 1 h at 4°C. To reduce background reactivity, the anti-rabbit IgGs were usually preabsorbed with heat-inactivated horse serum. After extensive washing with cold PBS, sections were fixed in cold 95% ethanol for 10 min, washed with PBS, and immersed for 30 s in 10 μg/ml of Hoechst Dye 33342 (Calbiochem-Behring Corp.). Washed sections were mounted with glycerol/PBS (9:1), sealed with colorless nail varnish, and stored at -20°C until observation. A microscope (Diaphot; Nikon Inc., Garden City, NY) equipped with epifluorescence optics was used for observation and microphotography of the sections.

To test for nonspecific fluorescence the following controls were used: (a) nonimmune, purified rabbit IgGs (Sigma Chemical Co.) at identical dilutions with the immune IgGs (1-10 μg/ml); (b) immune IgGs after passage through a [1-24]bFGF-Sepharose affinity column; and (c) immune IgGs (10 μg/ml) incubated with recombinant bovine bFGF (10 μg/ml) for 4 h at 4°C in the presence of 1% BSA-PBS and passed through a small heparin-Sepharose column (0.1 ml column bed volume for every 200 μl of incubation mixture). All controls used gave comparable results. A fluorescent image was considered to be bFGF-specific when it was obtained with the [1-24]bFGF-specific IgGs but not with any of the above mentioned controls. When immune IgGs were passaged through a keyhole limpet hemocyanin-Sepharose

affinity column, or through a heparin-Sepharose column, they retained the same reactivity as nonpassaged immune IgGs.

Results

Mitogenicity of Cardiac Extracts

Extraction of cardiac tissue with 2 vol of MEM (over tissue weight) resulted in ~1.5 vol of extract with a protein concentration of 15-17 mg/ml. MEM was used so that extracts could be tested immediately for biological activity on cells in vitro. Representative results are shown in Fig. 1, where extracts have been tested at a final concentration of 0.5 mg/ml. DNA synthesis as measured by [³H]thymidine incorporation was increased two- to fourfold in the presence of extracts compared with untreated controls. The apparent concentration of mitogenic activity is higher in the atrial extracts (compared with ventricular ones) in all species tested (chicken, rat, bovine, and sheep). Furthermore, extracts from chicken hearts appear to have more mitogenic activity overall than any of the mammalian extracts (Fig. 1). Actual levels of stimulation obtained varied somewhat between preparations; speed of extraction and short-term storage result in better reproducibility and more active preparations, especially for the atrial extracts. Rat tissue extracts were very susceptible to activity losses and they were used immediately after preparation.

The effect of cardiac extracts on myoblast differentiation was examined. Stimulation of proliferation in combination with inhibition of terminal differentiation of myoblasts would most likely reflect the presence of bFGF-like activity. Differentiation (presence of multinucleated myotubes) was assessed by determination of fusion index. A single addition of cardiac extracts was made at the time of plating at a final concentration of 0.5 mg/ml. Results are summarized in Table I: after 2.5 d, control cultures are differentiated, but all cultures in the presence of cardiac extracts are still proliferating. Fig. 2 shows characteristic results obtained in the presence of chicken extracts after 4 d; mammalian extracts produced identical results. In the presence of atrial extracts, cultures are still dominated by proliferating cells, while cultures treated with ventricular extracts are differentiated (Fig. 2 a and Table I). When ventricular extracts are supplemented with bFGF (10 ng/ml) their effect is similar to that of the unsupplemented atrial extracts (Fig. 2, a and c, and Table I). In contrast, if

Table I. The Effect of Chicken Cardiac Extracts on Skeletal Muscle Differentiation in Vitro*

Additions	Fusion index (day after addition of extract)		
	2.5	4	6
None (controls)	60	75	62
Atrial extract	12	15	74
Ventricular extract	27	80	70
Ventricular extract + bFGF (10 ng/ml)	15	15	70
Ventricular extract + IGF I (20 ng/ml)	40	85	76

* Extracts were added to the cells once at time of plating at a final concentration of 0.5 mg/ml. Fusion index = (nuclei inside myotubes/total number of nuclei) × 100; each value represents an average of four determinations (each determination varied by no more than 15% from the value shown).

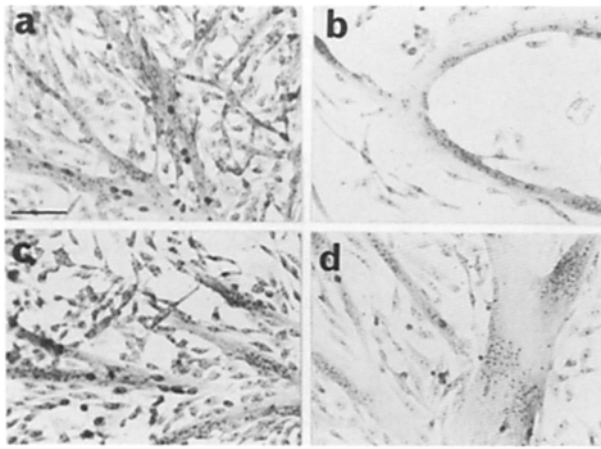


Figure 2. Morphology of chicken skeletal myoblast cultures grown in the presence of chicken cardiac extracts (final concentration of 0.3 mg/ml), with or without added growth factors, for 4 d. Cultures are fixed and stained with eosin and hematoxylin. Cultures grown with (a) atrial extract; (b) ventricular extract; (c) ventricular extract plus bFGF (10 ng/ml); and (d) ventricular extract plus IGF I (20 ng/ml). Bar, 50 μ m.

IGF I is added with the ventricular extracts, muscle differentiation is not delayed (Fig. 2, b and d, and Table I). If no more extract additions are made, all cultures are differentiated after 6 d (Table I).

A dose-dependent stimulation of myoblast [3 H]thymidine incorporation by chicken atrial and ventricular extracts is shown in Fig. 3. Stimulation by the atrial extracts is higher at all concentrations tested (0.1–1 mg/ml). Nevertheless, at

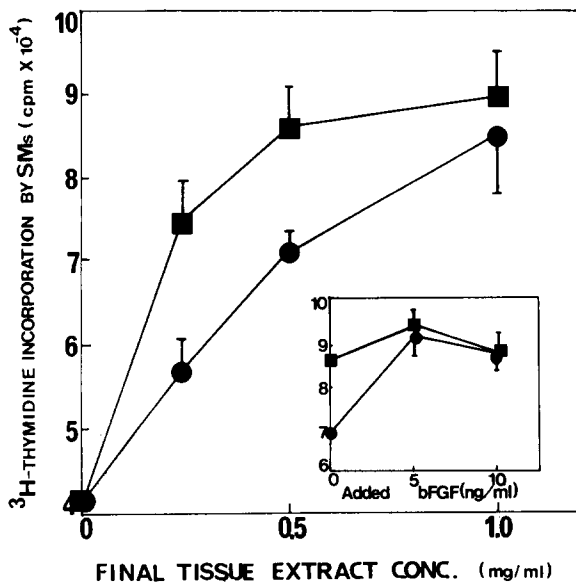


Figure 3. Dose-dependent stimulation of chicken skeletal myoblast DNA synthesis by chicken cardiac extracts after 24 h in culture. Squares and circles represent atrial and ventricular extracts, respectively. (Insert) Stimulation of chicken skeletal myoblast DNA synthesis (y-axis) by added bFGF (x-axis) in the presence of 0.5 mg/ml atrial (squares) or ventricular extract (circles). Values shown represent the mean of six determinations with the standard deviation indicated as a vertical line.

Table II. The Effect of the 2.5 M Heparin Column Eluates from Cardiac Tissue Extracts on Rabbit Fetal Chondrocyte DNA Synthesis

Additions	[3 H]Thymidine incorporation by rabbit fetal chondrocytes
	<i>cpm</i>
None	2,400 (200)
bFGF (0.5 ng/ml)	4,600 (350)
bFGF (2 ng/ml)	4,500 (450)
Bovine A*	4,800 (500)
Bovine V*	5,700 (550)
Rat A	ND
Rat V*	5,700 (600)
Sheep A	ND
Sheep V*	5,900 (600)
Chick A*	5,400 (450)
Chick V*	5,200 (550)

A and V, 2.5 M salt eluate from atrial and ventricular extracts, respectively. (*) Final concentration of 10 ng/ml. Values shown represent an average of six determinations with standard deviation from the mean shown in parentheses.

high extract concentrations, the difference in biological activity lessens (Fig. 3). Both atrial and ventricular extracts were supplemented with bFGF and tested for activity. 5 ng/ml of bFGF induced a small (8%) increase in atrial, but a sizeable (33%) increase in ventricular mitogenicity (Fig. 3, insert). At 10 ng/ml of added bFGF, both types of extracts exhibit identical activities, slightly lower than those at 5 ng/ml of added bFGF; we have previously seen this slight decrease at bFGF concentrations above those needed for saturation of the mitogenic response (26).

Identification of bFGF in Cardiac Extracts

We had found previously that cardiac extracts passed through heparin columns lose their mitogenic activity (23). The heparin-bound fraction from all extracts tested did indeed possess mitogenic activity for rabbit fetal chondrocytes as demonstrated by the results shown in Table II. Pure bFGF induces stimulation of a similar magnitude. This, together with evidence presented above, suggests strongly that bFGF is the peptide largely responsible for mitogenicity of cardiac extracts and can account for the differences between atrial and ventricular activities. Further indirect support for this claim comes from the use of TGF β -neutralizing antibodies with the extracts (Table III). The inhibitory action of pure human TGF β on cells is cancelled by neutralizing antibodies. When these antibodies are included with the cardiac extracts there is no change of extract mitogenicity (Table III), which strongly suggests that the difference between atrial and ventricular extracts is not caused by TGF β -like components. However, the possibility that endogenous TGF β -like reactivities do not recognize the neutralizing antibodies cannot be totally ruled out. To obtain positive evidence for bFGF presence and quantity in our extracts, we prepared antibodies against the [1-24]NH $_2$ -terminal peptide of bovine brain bFGF. Pure anti-[1-24]bFGF IgGs obtained by affinity chromatography were tested for specificity in immunoblots containing pure recombinant bFGF, aFGF, and serum or total crude cardiac extract protein. Pure immune IgGs (assayed at 20 ng/ml to 5 μ g/ml) recognized only bFGF. Similar results

Table III. The Effect of TGF β -neutralizing Antibodies on the Mitogenic Activity of Cardiac Extracts

Additions	[³ H]Thymidine incorporation by rabbit fetal chondrocytes
	cpm
None	2,400 (200)
TGF β (1 ng/ml)	1,700 (200)
Anti-TGF β (50 μ g/ml)	2,600 (300)
Rat atrial extract*	4,500 (450)
Rat ventricular extract*	3,700 (300)
Rat atrial extract* + anti-TGF β (50 μ g/ml)	4,100 (500)
Rat ventricular extract* + anti-TGF β (50 μ g/ml)	3,500 (450)

(* Final concentration of 0.25 mg/ml. Values shown represent an average of six determinations with standard deviation from the mean shown in parentheses.

were obtained with enzyme-linked immunoassay, using as antigens 50 ng/ml of pure, nondenatured bFGF, total serum, extract proteins, or keyhole limpet hemocyanin (50 μ g/well). A detailed characterization of the [1-24]bFGF IgGs will be presented elsewhere (Kardami, E., manuscript in prepara-

tion). We therefore consider the purified IgGs bFGF specific and, as such, they were used to identify bFGF-like peptides in the heparin-bound fractions. A silver-stained gel of some of these fractions is shown in Fig. 4 a, where an 18,000-D band, comigrating with pure bFGF, is clearly detected in atrial and ventricular heparin-binding fractions from chicken and cow (Fig. 4 a, lanes c-f). This band is recognized by the anti-[1-24]bFGF IgGs in all cardiac fractions tested (Fig. 4 b [chicken, lanes c and d; cow, lanes a and b]; c [chicken, lanes a and b; sheep, lanes c and d]; and d [chicken, lanes b and c; rat lanes d and e]). A lower molecular mass (~14,000-D) heparin-binding peptide seen in the silver-stained fractions and which may represent a truncated bFGF, as well as some larger peptides (Fig. 4 a), is not recognized by the antibodies. In all instances, atrial fractions contain more of the immunoreactive 18,000-D peptide (Fig. 4, a-d). This is illustrated clearly in Fig. 4 b, where the total heparin-binding fraction from 1 ml of chicken atrial (lane c) and ventricular (lane d) extract is shown which was obtained with a minimum of preparative losses (see Materials and Methods). The lowest amount of immunoreactive bFGF was seen in the rat extracts (Fig. 4 d, lanes d and e). Rat extracts were particularly sensitive to loss of biological activity upon handling, presumably due to degradation, and this may be the reason for the low levels of bFGF detected by the antibodies.

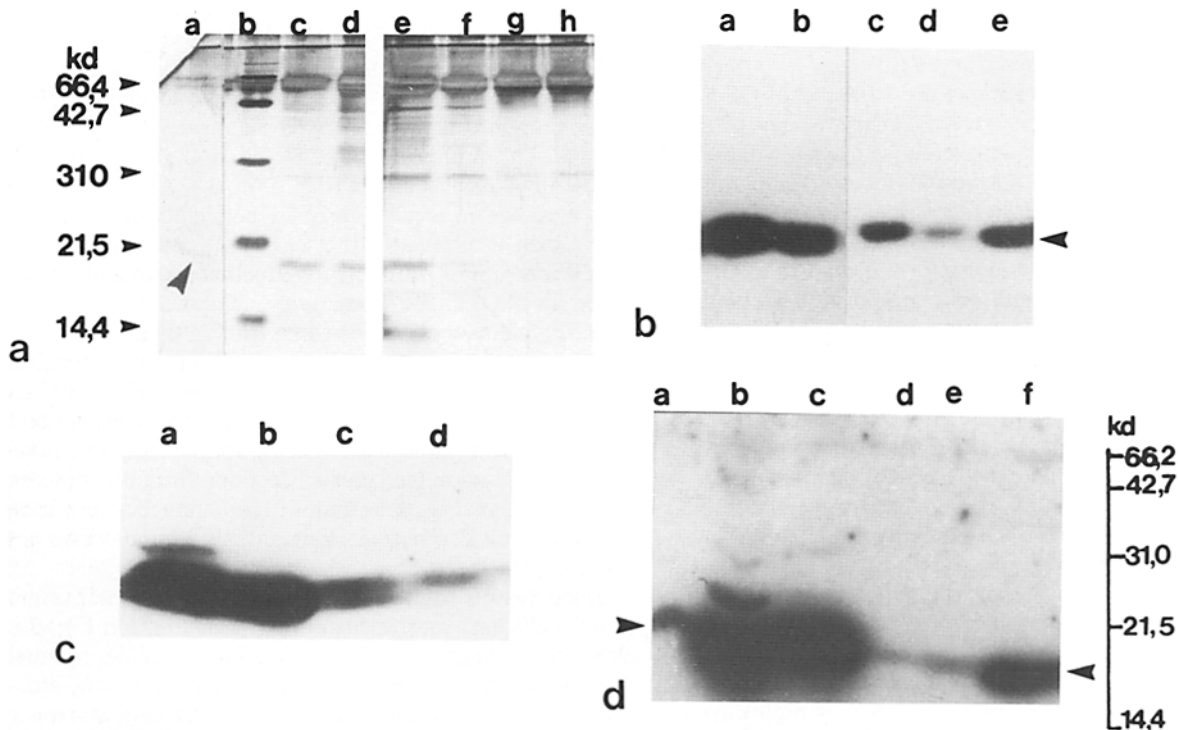


Figure 4. Identification of bFGF in cardiac extracts (chicken, rat, sheep, and bovine; protein content = 15 mg/ml). (a) Silver-stained gel with (lane a) 20 ng of recombinant bFGF; (lane b) relative molecular mass markers; and (lanes c-h) heparin-bound, 2 M salt eluates from cardiac tissue extracts. Eluate from 3.2 ml of chicken atrial extract contained ~50 ng protein (lane c); 6.4 ml of chicken ventricle extract contained 100 ng protein (lane d); 13 ml of bovine atria extract contained 150 ng protein (lane e); 17 ml of bovine ventricle contained 100 ng protein (lane f); 5 ml of rat atria contained 30 ng protein (lane g); and 10 ml of rat ventricle extracts contained 50 ng protein (lane h). (b-d) Autoradiograms of Western blots incubated with anti-[1-24]bFGF antibodies. (b) Heparin-bound, 2 M salt eluate from extracts from bovine atria (13 ml; lane a) and ventricle (17 ml; lane b); heparin-bound fraction from 1 ml of chicken atria (lane c) and ventricle (lane d); and recombinant bFGF (4 ng; lane e). (c) Heparin-bound eluate from extracts of chicken ventricles (10 ml; lane a) and atria (5 ml; lane b) and sheep ventricle (10 ml; lane c) and atria (5 ml; lane d). (d) Heparin-bound eluate from extracts of chicken ventricle (15 ml; lane b) and atria (10 ml; lane c); rat ventricle (10 ml; lane d) and atria (7 ml; lane e); and 5 and 10 ng recombinant bFGF (lanes a and f, respectively).

Table IV. Immunoreactive bFGF Concentration in Heart Tissues

Tissue	Amount bFGF per extracted tissue ng/g
Chicken atria	4.6
Chicken ventricles	1.4
Bovine atria	1.1
Bovine ventricle	0.4
Sheep atria	1.4
Sheep ventricle	0.5
Rat atria	0.3
Rat ventricles	0.1

Values shown represent the mean from three determinations (each from a different preparation). Each determination did not vary by more than 20% from the values shown.

Fractions from two different chicken preparations are shown in Fig. 4, *c* and *d*. A higher molecular mass (23,000-D) peptide, revealed by its reactivity with the antibodies, is always present in the ventricular but has not been seen in the atrial fractions. Storage did not have any detectable effect on its quantity (our unpublished observations). This peptide is considered to be bFGF-like because it binds heparin with high affinity and reacts with the anti-bFGF antibodies. It has been observed in all chicken ventricular preparations obtained so far. An additional bFGF-like peptide of ~17,000 D is also seen in ventricular but not atrial preparations from sheep hearts (Fig. 4 *c*, lanes *c* and *d*).

We have attempted to quantitate the 18,000-D bFGF-like peptides present in heart extracts by densitometry of autoradiograms obtained from immunoblots reacted with iodinated protein A (see Materials and Methods). We assumed that bFGF-like peptides from various tissues and species contain the same number of immunoreactive epitopes since bFGF is a highly conserved molecule and chicken bFGF is reported to have same amino acid composition as bovine bFGF (18). In addition, the amount of the 18,000-D heparin-bound peptide from all cardiac extracts examined, as seen in silver-stained gels (Fig. 4 *a* and our unpublished observations), agreed very well with the amount estimated by immunoreaction. As seen in Table IV, atrial extracts contain at least twice as much immunoreactive 18,000-D bFGF as ventricular, and avian extracts have at least three times the amount of mammalian extracts. It is expected that values shown in Table IV represent a lower estimate of the actual amount of bFGF present in cardiac compartments because of preparative losses and probable degradation during handling. It is also conceivable that active bFGF-like peptides may be present in different tissues that are not recognized by our antibodies. This is unlikely since bFGF is highly conserved (18–20) and biological activity data (Fig. 1) agree well with the approximate quantitation obtained from the immunoblots.

It is possible that the observed differences in bFGF concentration in the extracts reflect real differences in the amount of bFGF present in vivo in various tissues. However, differences in tissue "extractability" could also account for differences in the amount of bFGF in the extracts and cannot be ruled out at this point; as will be shown in the following section, in at least one case (bovine atria vs. bovine ventricle), visualization of bFGF-like reactivities in vivo agrees

well with the result obtained from bFGF quantitation in extracts.

Localization of bFGF in Bovine Cardiac Tissue Sections by Indirect Immunofluorescence

Our anti-[1-24]bFGF antibodies are highly specific for bFGF in immunoblots and enzyme-linked immunoassays. They recognize a part of the bFGF molecule at the edge of one of the heparin-binding domains (6). These IgGs therefore might bind bFGF even if it is "trapped" by heparin-rich areas in its physiological environment. Anti-[1-24]bFGF IgGs may recognize common sequences present in non-bFGF peptides. This is not very likely since they do not react with any other peptide in crude bovine tissue extracts. When pure IgGs are absorbed with native bFGF they lose their reactivity completely. Staining observed by indirect immunofluorescence is therefore considered bFGF specific.

We investigated the immunoreactivity of bovine atrial and ventricular sections, focusing primarily on cardiac myocytes. Cardiac muscle was identified by simultaneous labeling of sections with antibodies specific for striated muscle myosin; nuclei were identified by staining with the fluorescent dye Hoechst 33342. Fig. 5, *a-c*, shows a characteristic triple-stained section from ventricular tissue and is used to illustrate the fact that muscle nuclei bind the anti-[1-24]bFGF IgGs (Fig. 5, *a-c*, shows bFGF immunofluorescence, myosin immunofluorescence, and nuclear fluorescence, respectively). Triple fluorescence staining was used to ensure that the bFGF immunoreactive nuclei belonged to muscle as well as nonmuscle cells. Muscle nuclei appeared to accumulate bFGF in association with their membranes, as seen most clearly in Fig. 5 *d*. They were recognized by the antibodies in both ventricular and atrial sections (Fig. 5, *a*, *d-f*, *h*, and *i*). Atrial myocytes seem to accumulate bFGF pericellularly to a greater extent than ventricular myocytes (Fig. 5, compare *a* and *d* with *e* and *f* and *h* with *i*). Longitudinal sections were also tested for bFGF immunoreactivity: as shown in Fig. 6, *a*, *b*, *e*, and *f*, the intercalated disc area from both atrial and ventricular fibers exhibits very bright fluorescence. Sections treated with non-bFGF-specific IgGs (see Materials and Methods), at concentrations similar to the specific antibodies (2–10 $\mu\text{g/ml}$), always presented a very weak fluorescence of the same region (see control sections from two different experiments in Fig. 6, *c* and *d*). Immunoreactive muscle nuclei can be also seen in longitudinal sections (Fig. 6, *e* and *f*).

Blood vessels stain brightly (Fig. 5 *e*) in all their layers. Single cells near muscle fibers (Fig. 7 *a*; Fig. 6 *f*; Fig. 5 *e*) show pericellular bFGF accumulation. In addition, small populations of cells (groups of two to six) in the interstitial space show particularly bright immunostaining as seen in Fig. 7 *c*; muscle nuclear immunostaining, although present, is not discerned in this picture which has been exposed for the brightness of the group of nonmuscle cells shown. Capillaries (endothelial cells) between muscle fibers also appear to accumulate bFGF (Fig. 5, *h* and *i*, large arrowheads).

In sections from ventricular myocardium nearest to the subepicardial layer we consistently (preparations from four different bovine hearts) observe a structure which stains brilliantly with the [1-24]bFGF antibodies (Fig. 8). This structure is found encased between muscle fibers (Fig. 8, *a* and *d*), does not react with myosin antibodies (not shown), is

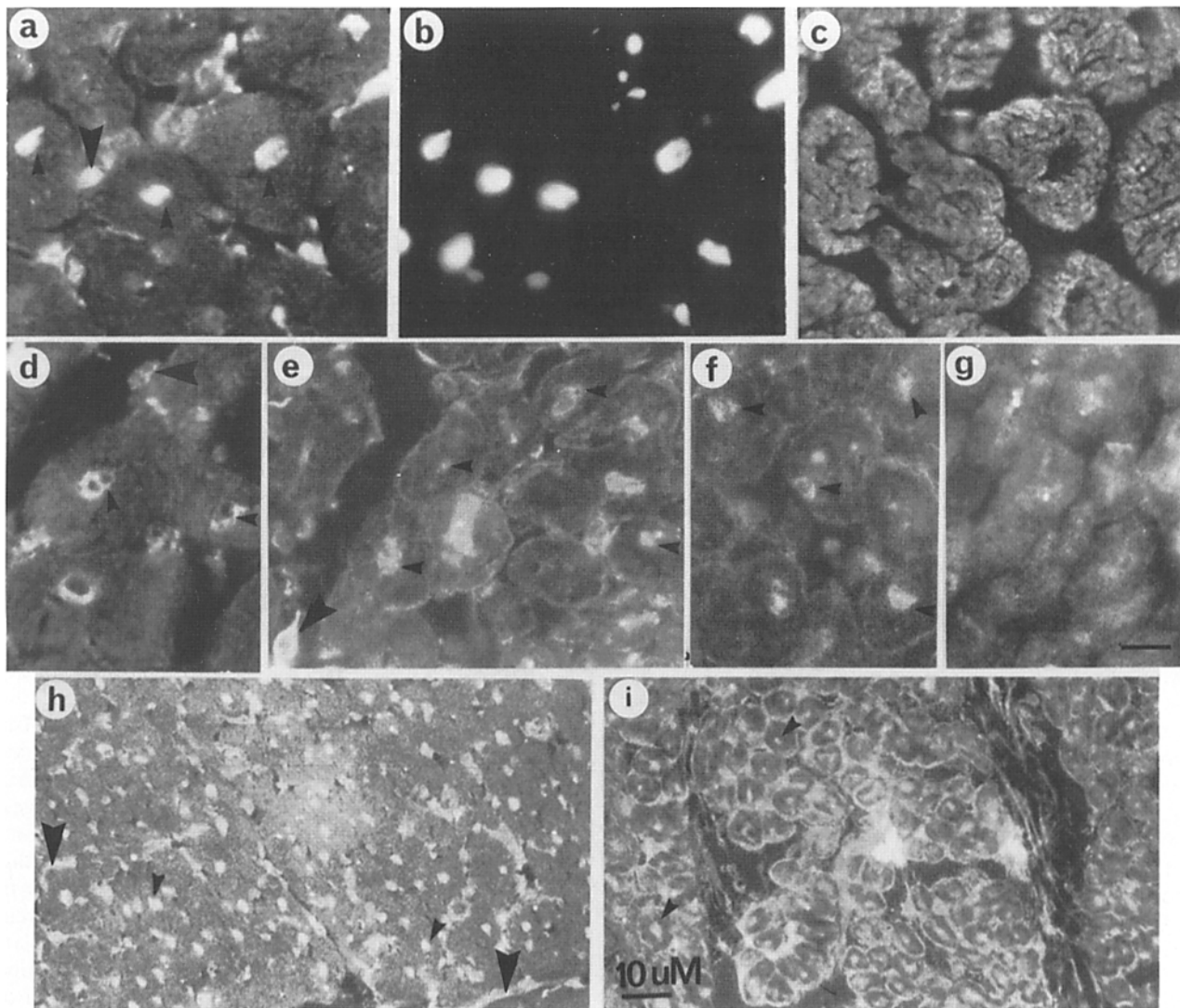


Figure 5. Localization of bFGF (*a*, *d-f*, *h*, and *i*) in muscle and nonmuscle cells in sections of bovine ventricular (*a-d*, *g*, and *h*) and atrial (*e*, *f*, and *i*) tissue at high (*a-g*) and low magnification. Direction of sectioning was at approximately right angles to the long axis of muscle fibers. Double immunofluorescence stain for bFGF (*a*), muscle myosin (*c*), and Hoechst 33342 nuclear stain (*b*) identifies muscle fibers, nonmuscle cells, and nuclei and shows nuclear localization of bFGF. Staining with non-bFGF-specific rabbit IgGs is shown in *g* (overexposed photograph). Perinuclear accumulation of bFGF is seen clearly in *d*. Pericellular staining is shown in *e*, *f*, and *i*. Small arrowheads mark muscle nuclei, while large arrowheads point at nonmuscle, bFGF-specific staining. Compare bFGF distribution between atria and ventricles in *h* and *i*; Bars: (*a-g*) 6 μm ; (*h* and *i*) 10 μm .

roughly egg shaped, has a width of 60–100 μm and length of ~ 200 μm , binds hematoxylin with high affinity (Fig. 8 *c*), and contains many nuclei (Fig. 8 *b*). One to two of these structures are encountered per square centimeter of subepicardial tissue. The identity of this structure is currently under investigation.

Discussion

Because bFGF has been strongly implicated with tissue repair *in vivo* (5, 11, 17–20), we examined cardiac atrial and ventricular extracts for bFGF-like activities as part of an effort to understand differences in regenerative properties of cardiomyocytes (47). Rabbit fetal chondrocytes and embryonic avian myoblasts, which are both highly responsive

to bFGF (25–27, 44), were used in indirect biological activity assays. In both instances, mitogenic stimulation was more pronounced in the presence of atrial extracts from all animals tested. In addition, atrial extracts delayed skeletal muscle differentiation in a manner identical to that of pure bFGF (25). These data suggested that differences in extract activity could be accounted for by differences in bFGF content. The activity of cardiac extracts remained unaffected by TGF β -neutralizing antibodies, which suggests that if this factor is present it is at concentrations too low to have an effect on extract biological activity. IGFs are also found in heart tissue (12). Heparin absorption of the extracts nevertheless results in an almost complete loss of mitogenicity extracts (23); this implicates a heparin-binding mitogen rather than an IGF activity. In addition, IGFs stimulate skeletal muscle differentia-

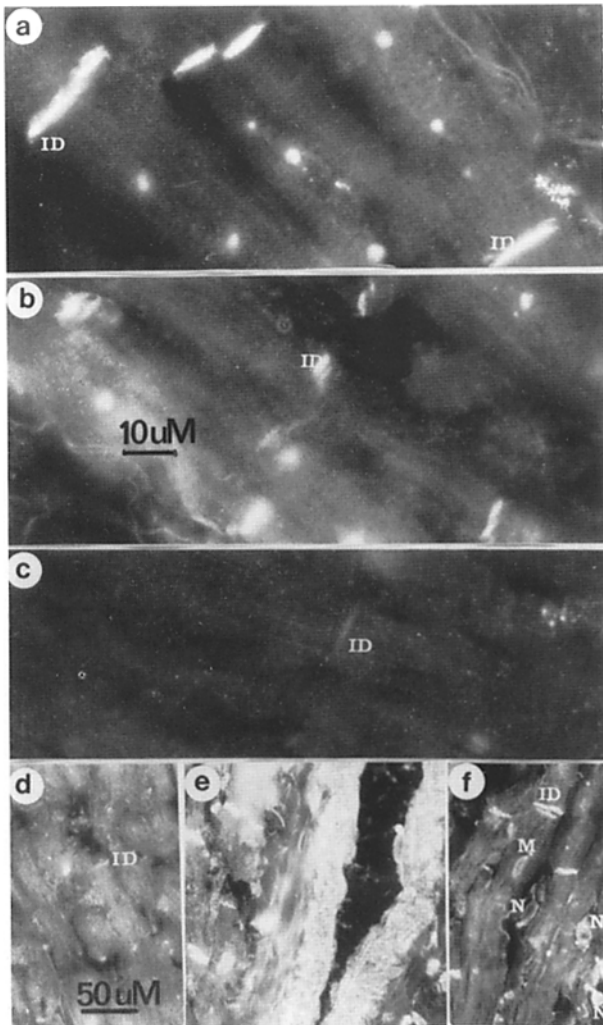


Figure 6. Localization of bFGF-like reactivities (*a, b, e, and f*) in cardiac muscle intercalated disc region (*a, b, e, and f*), cardiac muscle nuclei (*e and f*), blood vessels (*e*), and interstitial cells (*f*). Sections were cut parallel to the long axis of the fibers. Immunofluorescence stain for bFGF in sections from bovine ventricles (*a and c-f*) and atria (*b*). Control stain shown in *c and d* (nonimmune rabbit IgGs and bovine whole bFGF-absorbed immune IgGs, respectively). *ID*, intercalated discs; *M*, muscle nuclei; and *N*, non-muscle cell. Fluorescein-conjugated IgGs in *d-f* were not preincubated with heat-inactivated horse serum. Bars: (*a-c*) 10 μm ; (*d-f*) 50 μm .

tion (14) so, if they are present in our extracts, their effect is masked by bFGF-like activities that have an apparently opposite effect.

Another heparin-binding factor that coexists with bFGF in some tissues is aFGF (19). Polyclonal aFGF-specific antibodies show no reactivity with the heparin-bound cardiac fractions eluting at 1.1 M salt (our unpublished observations). We therefore conclude that the major mitogen in our cardiac tissue extracts is a bFGF-like activity, being more abundant in atrial extracts. Direct evidence for this claim was obtained by fractionating extracts by heparin affinity chromatography and testing the 2.5 M salt eluates for biological activity and reactivity with anti-bFGF IgGs. An immunoreactive 18,000-D peptide was identified in all cases, comi-

grating with recombinant bovine bFGF. The amount of this peptide was always higher in the atrial extracts, confirming results from biological assays. It is therefore tempting to correlate higher concentrations of bFGF in the atria with the proliferative and regenerative properties of atrial myocytes. Recent findings by us and others show that bFGF does stimulate embryonic or adult cardiomyocyte DNA synthesis as well as embryonic cardiomyocyte proliferation *in vitro* (9, 21, 22).

Chicken extracts from either ventricles or atria had the highest concentration of immunoreactive bFGF among the species examined. The 23,000-D bFGF-like peptide in chick ventricular extracts may represent a precursor of the predominating 18,000-D form. Its presence in small amounts in ventricular but not atrial extracts could be explained by increased proteolytic activity in the atrial extracts. The NH_2 -terminal portion of bFGF is very susceptible to limited proteolysis by naturally occurring proteases (29) and higher molecular mass bFGF peptides do exist in embryonic tissue that can produce the 18,000-D form after digestion (32). Whatever the relationship between the 23,000- and 18,000-D peptides may be, it is interesting that the 23,000-D peptide is present in embryonic atrial extracts and it increases in quantity in embryonic ventricular extracts (Kardami, E., manuscript in preparation).

An important question that has been addressed indirectly so far, concerns the exact localization of bFGF within tissues, which may provide a clue for its function *in vivo*. The high affinity of this peptide for heparin and heparan-sulfate (17), its accumulation in tissues rich in extracellular matrix (4, 5, 25), and the relative ease for its extraction suggest strongly that bFGF is an extracellular matrix component. Immunofluorescence of cardiac tissue sections supports this notion, particularly for the atrial myocardium. Atrial myofibers appear surrounded by bFGF-rich endomysium. Ventricular myofibers are much more compact and accumulate less bFGF pericellularly. This difference, in combination with the smaller size of atrial fibers (more intercalated discs and more nuclei per unit mass), is most likely contributing to higher bFGF concentrations in the atrial extracts. The significance of bFGF accumulation around atrial myofibers is as yet unknown. It is tempting to speculate that it may have an effect on atrial cell proliferative properties.

The presence of bFGF inside atrial and ventricular muscle cells and in association with the nuclear membrane is intriguing since it suggests that bFGF is synthesized by the myocytes. Many cell types that are responsive to bFGF synthesize this peptide in culture (20, 45), implying an autocrine mode of growth regulation. The source(s) of bFGF accumulated *in vivo* is nevertheless unknown. Since tissues with a high degree of vascularization also contain high levels of bFGF, endothelial cells are believed to be the source of this factor (18, 20). Our data suggest that other cell types, such as cardiac myocytes, are candidates for bFGF production *in vivo*. This is an attractive notion since cardiac muscle differentiates very early during embryonic development, before any vessels are formed. Release of bFGF activities by the myocytes could be a trigger for formation of capillaries and vessels in the heart, as well as for promoting cardiac innervation (39, 46).

The presence of bFGF-like components in the intercalated disc areas of the myocytes suggests an involvement with cell-cell recognition, attachment, and communication. This

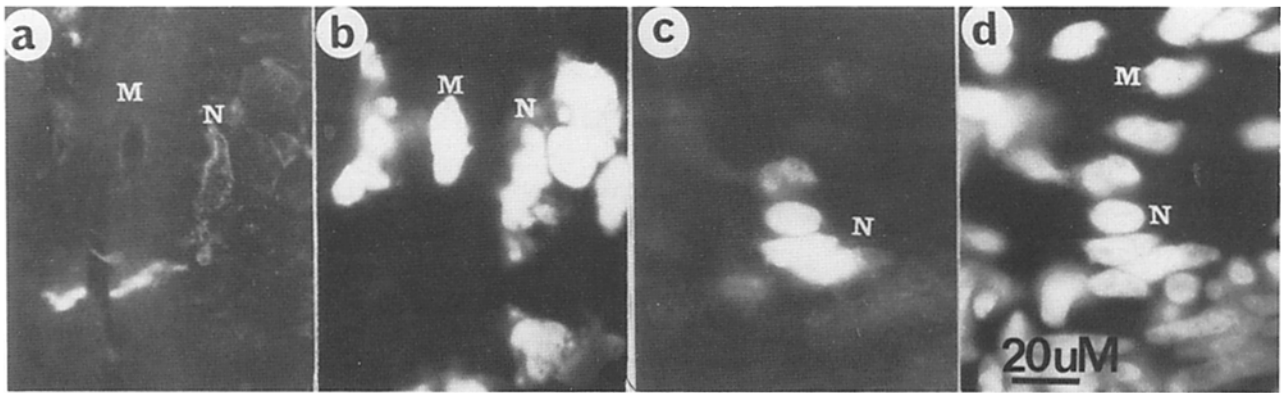


Figure 7. bFGF in interstitial cells (a and c). Double staining for bFGF (a and c; immunofluorescence) and nuclei (b and d; Hoechst 33342) in longitudinal sections from bovine ventricles. M, muscle nuclei, N, nonmuscle cells. Bar, 20 μm .

has been already predicted from the amino acid sequence of bFGF which contains cell recognition sequences (13). In addition, bFGF was shown to influence cell-substratum adhesion in a sympathetic nerve cell line (39).

Our observations have been focused mainly on striated muscle cells in the heart. bFGF nevertheless was accumulated by other cell types. Blood vessels show accumulation in all their layers, in association most likely with their heparan-sulfate-rich extracellular matrix (4). Certain connective tissue cells also stain very brightly with the anti-bFGF IgGs. This is not unexpected since connective tissue contains mast cells, macrophages, or fibroblasts, all of which probably contain bFGF in vivo.

Could bFGF be recruited in inducing cardiomyocyte regeneration? It has been speculated that degradation of extracellular matrix by enzymes released through the immune

response would liberate bFGF which could then stimulate cell proliferation within the available space (4). Nonmuscle cells would probably respond faster and no significant myocyte regeneration would occur before damaged tissue was replaced by scar tissue. If, however, the action of bFGF on nonmuscle cells could be slowed and that on muscle cells promoted to the point of actual cell division, this might result in effective muscle regeneration or at least reduction of scarring. There is evidence that regulation of proliferation differs depending on cell type. TGF β , for example, stimulates fibroblast proliferation while it cancels the action of bFGF on endothelial cells, skeletal myoblasts (14, 43), and cardiac neonatal myocytes (22, 23). Since TGF β , as well as bFGF, is present in the heart (35), effective inhibition of its action may be a way of stimulating myocyte, while delaying fibroblast, proliferation. Further studies on the control of growth

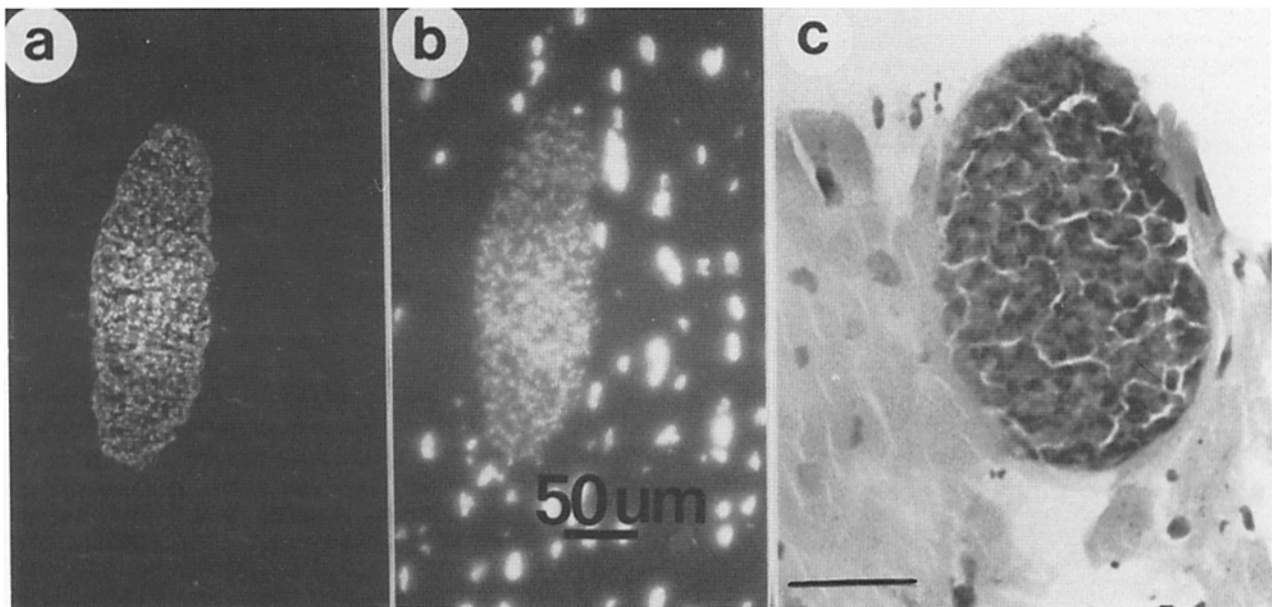


Figure 8. bFGF in an unidentified structure in the bovine ventricle (a and b). Double fluorescence staining for bFGF (a) and nuclei (b) in longitudinal section. Histochemical staining (eosin-hematoxylin) of similar structure (c) shows strong affinity for hematoxylin. Bars: (a and b) 50 μm ; (c) 50 μm .

factor expression, secretion, and accumulation by different cell types will be of invaluable assistance in understanding the processes involved in tissue regeneration.

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