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A growth factor for cardiac myocytes is produced by cardiac nonmyocytes

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Cardiac nonmyocytes, primarily fibroblasts, surround cardiac myocytes in vivo. We examined whether nonmyocytes could modulate myocyte growth by production of one or more growth factors. Cardiac myocyte hypertrophic growth was stimulated in cultures with increasing numbers of cardiac nonmyocytes. This effect of nonmyocytes on myocyte size was reproduced by serum-free medium conditioned by the cardiac nonmyocytes. The majority of the nonmyocyte-derived myocyte growth-promoting activity bound to heparin-Sepharose and was eluted with 0.75 M NaCl. Several known polypeptide growth factors found recently in cardiac tissue, namely acidic fibroblast growth factor (aFGF), basic FGF (bFGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNFα), and transforming growth factor β1 (TGFβ_1), also caused hypertrophy of cardiac myocytes in a dose-dependent manner. However, the nonmyocyte-derived growth factor (tentatively named NMDGF) could be distinguished from these other growth factors by different heparin-Sepharose binding profiles (TNFα, aFGF, bFGF, and TGFβ_1) by neutralizing growth factor-specific antisera (PDGF, TNFα, aFGF, bFGF, and TGFβ_1), by the failure of NMDGF to stimulate phosphatidylinositol hydrolysis (PDGF and TGFβ_1), and, finally, by the apparent molecular weight of NMDGF (45–50 kDa). This nonmyocyte-derived heparin-binding growth factor may represent a novel paracrine growth mechanism in myocardium.

Introduction

The complexity of cellular organization in tissues suggests a role for cell–cell interactions in both cell and tissue function. The heart, for example, functions as a syncytium of myocytes and the surrounding support cells, collectively referred to as nonmyocytes. These nonmyocytes consist predominantly of fibroblast/mesenchymal cells but include endothelial and smooth muscle cells (Nag, 1980). In fact, although myocytes make up most of the adult myocardial mass, they comprise only ~30% of the total cell numbers present in heart (Zak, 1973). Because of their intimate relationship with cardiac myocytes in vivo, these nonmyocytes have the potential to influence myocyte growth and/or development. This influence could occur directly through cell–cell contact or indirectly via the production of a paracrine factor or factors. The potential importance of such an interaction in vivo is emphasized by the observation that both nonmyocyte numbers and the extracellular matrix they elaborate are increased in myocardial hypertrophy and in response to injury and infarction (Morkin and Ashford, 1968; Carroll *et al.*, 1989; Huysman *et al.*, 1989; Weber *et al.*, 1989; Chapman *et al.*, 1990). These changes are associated with abnormal myocardial function (Carroll *et al.*, 1989; Brilla *et al.*, 1991).

Soon after birth, cardiac myocytes lose the ability to undergo cell division. Further growth occurs through hypertrophy of the individual cells. In an effort to elucidate the molecular mechanisms regulating cardiac myocyte hypertrophy, cell culture models of myocyte hypertrophy have been established (Simpson *et al.*, 1982). To isolate the actions of potential growth-promoting agents to the myocytes themselves, most studies of heart myocytes in culture have sought to minimize nonmyocyte contamination (Simpson and Savion, 1982; Simpson *et al.*, 1982; Libby, 1984; Simpson, 1985). Although this model has increased our understanding of some of the mechanisms of myocyte hypertrophy (Simpson, 1989), the effect of the nonmyocytes on myocyte growth in culture has never been examined adequately.

We first investigated the effect of the cardiac nonmyocytes on cardiac myocyte growth in culture. Myocyte hypertrophic grown was stimulated in high-density cultures with increased numbers of nonmyocytes and in cocultures with increased numbers of nonmyocytes. This effect

of nonmyocytes on myocyte size could be reproduced by serum-free medium conditioned by nonmyocyte cultures (NMC-CM),[1] suggesting the presence of a paracrine factor or factors. Several previously described growth factors bind to heparin (Gospodarowicz *et al.*, 1984; Klagsbrun and Shing, 1985; Delli-bovi *et al.*, 1988; Ferrara and Henzel, 1989; Finch *et al.*, 1989; Klagsbrun, 1989; Tischer *et al.*, 1989; Besner *et al.*, 1990). The major myocyte growth-promoting activity in NMC-CM was also heparin-binding, eluting with 0.75 M NaCl. The properties of this heparin-binding nonmyocyte-derived growth factor were compared with those of the growth factors found by Northern blot and immunoaffinity analysis to be present in myocardium, namely acidic fibroblast growth factor (aFGF), basic FGF (bFGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNFα), and transforming growth factor β1 (TGFβ_1). All five of these growth factors also caused myocyte hypertrophy in culture. However, their heparin-Sepharose elution profiles suggested that the nonmyocyte-derived factor was not aFGF, bFGF, TNFα, or TGFβ_1. Furthermore, despite a heparin-Sepharose elution profile similar to that of PDGF, the nonmyocyte-derived growth factor described here was not neutralized by a PDGF-specific antibody and appeared to act via a different second messenger system in that it failed to stimulate phosphatidylinositol hydrolysis. In addition, the apparent molecular weight of this nonmyocyte-derived growth factor (45–50 kDa) was larger than that of any of these known growth factors. Thus, this nonmyocyte-derived factor is potentially unique and may define a paracrine relationship important for cardiac muscle cell growth and development.

Results

Myocyte size increases with increasing cell density

The possibility that autocrine or paracrine factors might be involved in growth of cardiac myocytes in culture was first suggested by the observation that myocyte size was increased as a function of cell density in the myocyte cultures. For these studies, myocyte cultures were prepared with increasing cell density (10–400 cells/mm^2). Myocytes appeared larger at higher cell densities (Figure 1A), and there was a linear relationship between cell protein content and cell density over the range tested (Figure 1B). Thus, myocytes in high-density cultures were larger than those in lower-density cultures. The increase in cell density in these experiments involves an absolute increase in the numbers of both myocytes and nonmyocytes because the fraction of nonmyocytes was constant at all densities (~10%). Therefore, increased myocyte size with increased cell density in culture could have reflected autocrine or paracrine growth factors or direct cell–cell interactions.

Myocyte size is increased by coculture with nonmyocytes

To explore the possible effect of nonmyocytes on myocyte size, cocultures were prepared with a constant number of myocytes and increasing numbers of cardiac nonmyocytes. These cardiac nonmyocytes in culture are probably fibroblasts (see Materials and methods). Myocytes in cocultures containing 37% nonmyocytes appeared larger than myocytes in the control cultures containing 10% nonmyocytes (Figure 2A). This hypertrophic effect was quantified by measuring myocyte surface area because total protein content in dishes with varying numbers of nonmyocytes would be a poor representation of myocyte protein. There was an increase in myocyte surface area as the number and percent of nonmyocytes in the cocultures were increased (Figure 2B). After 5 d, myocyte size measured by surface area was increased by ~1.3-fold versus control in the cocultures containing 37% nonmyocytes (2423 ± 83 [mean ± SE] μm^2 per cell in cocultures vs. 1926 ± 65 μm^2 per cell in control cultures with 9.6% nonmyocytes, n = 100 cells, $p < 0.001$). Significant increases in myocyte size were evident by 2 d in coculture (data not shown).

NMC-CM increases myocyte size

The increased myocyte size observed in cocultures with increased nonmyocytes could have been due to either a direct nonmyocyte/myocyte interaction or a paracrine growth factor or fac-

[1] Abbreviations used: aFGF and bFGF, acidic and basic fibroblast growth factors; BrdU, bromodeoxyuridine; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; MEM, (Eagle's) minimum essential medium; MEM/TI, MEM supplemented with transferrin and insulin; MEM/TIB-BSA, MEM supplemented with transferrin, insulin, BrdU, and bovine serum albumen; NMC-CM, nonmyocyte-conditioned medium; NMDGF, nonmyocyte-derived growth factor; PDGF, platelet-derived growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGFβ1, transforming growth factor β1; TNFα, tumor necrosis factor α; Tris, tris(hydroxymethyl)aminomethane.

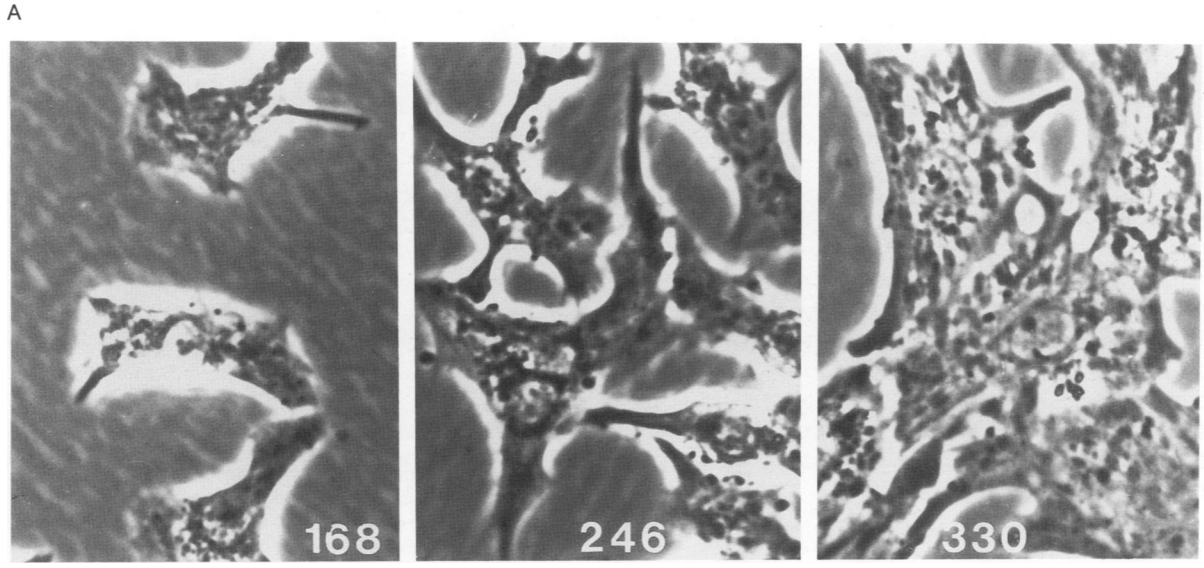


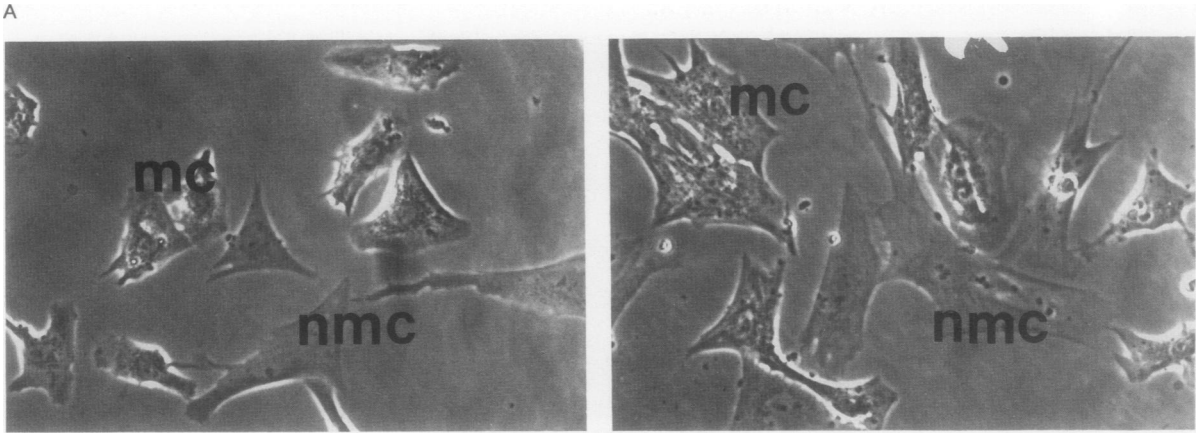
Figure 1. **Increasing cell density results in increased myocyte size.** Cardiac myocytes were isolated from neonatal rat hearts and cultured in serum-free medium, as described in Materials and methods, at increasing final densities from 10 to 400 cells/mm^2. (A) Photomicrograph of myocytes at three different plating densities. Note the increased size of myocytes plated at 330 cells/mm^2 compared with those plated at 168 cells/mm^2. (B) Graph of cell size (described as radiolabeled protein content in dpm per 1000 cells) vs. cell density. Cardiac myocyte size increased in a nearly linear fashion over the densities studied ($r = 0.9$). All cultures contained ~10% nonmyocytes. Each point is mean ± SE for three dishes in a single experiment. Similar results were obtained in two additional experiments.

tors. Therefore, NMC-CM was tested for myocyte growth-promoting activity.

Treatment of the cultured myocytes with NMC-CM (50% vol/vol) increased myocyte surface area by ~1.5-fold versus that of control myocytes treated with fresh Eagle's minimum essential medium supplemented with transferrin and insulin (MEM/TI) ($p < 0.001$, Table 1). In addition, myocyte protein content was increased by ~1.4-fold versus control ($p < 0.001$, Table 1). This increase in myocyte protein was dose dependent with a significant effect at 5% NMC-CM and a plateau at 50% NMC-CM (vol/vol) (Figure 3).

No significant change in myocyte size was produced when the cells were treated with medium conditioned by cultured myocytes (1×10^6 cells per dish, ~10% nonmyocytes) or with mock-conditioned medium processed in dishes without cells (Table 1). Because these control conditioned media were prepared in the same manner as NMC-CM, this result suggested that the presence of growth-promoting activity in conditioned medium required nonmyocytes.

The growth-promoting activity in NMC-CM increased linearly as the number of nonmyocytes in the conditioning dishes was increased from 2.5×10^5 per dish to 1×10^6 per dish (Figure 4A). The presence of growth-promoting activity in conditioned medium increased with conditioning time (Figure 4B). Two days of conditioning were required for a significant effect



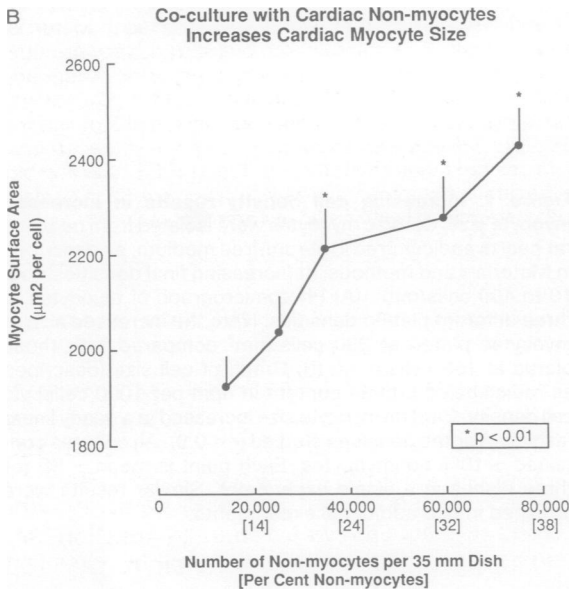


Figure 2. **Coculture with cardiac nonmyocytes increases cardiac myocyte size.** (A) Cardiac myocytes were cultured using the usual protocol (left) or cocultured with additional nonmyocytes (right). After 5 d in serum-free MEM/TI, cell numbers were determined and photomicrographs were obtained. BrdU was present for the first 3 culture days. Both cultures contained $9.5 \pm 0.2 \times 10^4$ myocytes per dish (119 per mm^2). In the normal control cultures (left), nonmyocyte number was $1.1 \pm 0.3 \times 10^4$ per dish (14 per mm^2, 10.4% of total cells). In the cocultures (right), nonmyocyte number was $5.7 \pm 0.2 \times 10^4$ per dish (71 per mm^2, 37.5% of total cells). Note the apparent increase in myocyte surface area in the cocultures with increased numbers of nonmyocytes (right). MC, myocyte; NMC, nonmyocyte. (B) Cardiac myocytes were cocultured as described above. Cell numbers were determined after 2 and 5 d in culture and the surface areas of all myocytes in randomly selected microscopic fields were quantified after 5 d in culture. There was no change in myocyte or nonmyocyte numbers over time in culture. All dishes contained the same number of myocytes ($1.28 \pm 0.06 \times 10^5$ per dish, 160 per mm^2). Nonmyocyte numbers per dish are given on the Y axis, with the percent nonmyocytes in brackets. Nonmyocyte number per dish ranged from 1.4×10^4 (18 per mm^2, 9.6% of total cells) in the normal control cultures to 7.6×10^4 (95 per mm^2, 37.4% of total cells) in the cocultures with the greatest number of nonmyocytes. Each data point is the mean myocyte surface area ± SE (n = 100 cells). *$p < 0.01$ vs. control cultures with 9.6% nonmyocytes.

of conditioned medium on myocyte size (Figure 4B). Medium conditioned for 3 d by $\sim 1 \times 10^6$ nonmyocytes was used for all subsequent experiments.

Experiments designed to investigate the physical properties of the factor(s) present in NMC-CM revealed no growth-promoting activity of NMC-CM (50% vol/vol) that had been heated (2 experiments), treated with trypsin (1 experiment), or reduced with 50 mM dithiothreitol (DTT) (2 experiments, testing the 0.75 M heparin-Sepharose fraction) (data not shown). These findings were consistent with the presence of a protein or proteins in NMC-CM that caused myocyte growth.

Nonmyocyte protein synthesis is required for NMC-CM growth-promoting activity

The progressive accumulation over time of myocyte growth-promoting activity in NMC-CM suggested that a factor or factors was being produced by the nonmyocytes and released into the medium.

Using the protein synthesis inhibitor cycloheximide, protein synthesis was also found to be required for the presence of growth-promoting activity in NMC-CM. Nonmyocyte cultures were treated for 3 d during conditioning with a concentration of cycloheximide (2 μM) that reduced nonmyocyte protein synthesis by

74%, as measured by incorporation of ^{14}C-phenylalanine into protein. This dose of cycloheximide did not change nonmyocyte numbers. NMC-CM from cultures treated with or without 2 μM cycloheximide were collected and fractionated by heparin-Sepharose chromatography (see below). Preliminary experiments showed that cycloheximide was not retained on the heparin-Sepharose column. (Mock-CM without or with 2 μM cycloheximide was applied to a heparin-Sepharose column and eluted with NaCl as for NMC-CM. Fractions were collected and tested for their effect on myocyte protein synthesis. In the 0.75 M fraction, the mock-CM with cycloheximide:mock-CM without cycloheximide ratio for protein synthesis was 1.09 ± 0.05, p = NS, n = 3.) Myocyte protein content was increased by 1.38 ± 0.04-fold versus control by the 0.75 M NaCl fraction of medium conditioned by nonmyocytes in the absence of cycloheximide. In contrast, myocyte protein content was increased by only 1.13 ± 0.08-fold versus control by the 0.75 M NaCl fraction of medium conditioned by nonmyocytes in the presence of cycloheximide (n = 3). This 72% reduction in the growth-promoting activity of the 0.75 M NaCl fraction was similar to the 74% reduction in overall protein synthesis produced by cycloheximide. This result suggested that cycloheximide had inhibited production of a growth factor or factors by the nonmyocytes.

NMC-CM contains a heparin-binding growth factor or factors

Several known growth factors bind to heparin, including aFGF, bFGF, PDGF, and vascular endothelial growth factor (Gospodarowicz *et al.*, 1984; Klagsbrun and Shing, 1985; Delli-bovi *et al.*, 1988; Ferrara and Henzel, 1989; Finch *et al.*, 1989; Klagsbrun, 1989; Tischer *et al.*, 1989; Besner *et al.*, 1990). Therefore, NMC-CM was analyzed by heparin-affinity chromatography. NMC-CM (50–100 ml/batch) was concentrated by ultrafiltration and applied to a 1-cm heparin-Sepharose column. The growth-promoting activity of NMC-CM before heparin-Sepharose binding were compared with the heparin-Sepharose flow-through. Seventy percent of the growth-promoting activity was retained on the heparin-Sepharose column (n = 5). With step-gradient NaCl elution, two peaks of growth-promoting activity were found. One eluted at 0.75 M NaCl and contained 80% of the retained activity. The remainder of the bound activity eluted at 1.5 M NaCl (Figure 5).

Table 1. Medium conditioned by cardiac nonmyocytes increases cardiac myocyte size

Medium	Myocyte size (fold-increase)	
	Surface area	Protein content[a]
Fresh nonconditioned (control)	1.0	1.0
Mock-conditioned	1.09	1.08 ± 0.06 (4)
Myocyte-conditioned		1.09 ± 0.04 (5)
Nonmyocyte-conditioned (NMC-CM)	1.51[b]	1.42 ± 0.04 (26)[b]

Beginning on culture day 1, cardiac myocytes in 35-mm culture dishes were maintained in 1 ml fresh nonconditioned culture medium (MEM with transferrin, insulin, and BrdU) or in 1 ml fresh medium/conditioned medium (50% vol/vol). Medium was conditioned for 3 d in empty dishes (mock-conditioned), by cardiac myocytes (myocyte-conditioned), or by cardiac nonmyocytes (nonmyocyte-conditioned). Myocyte surface area and protein content were determined after 3 d in the different media, as described in Materials and methods. The surface area of 100 randomly-selected myocytes was quantified in a single experiment. Surface area was 1493 ± 72 μm^2/cell (mean ± SE) for control myocytes maintained in fresh nonconditioned medium and the SE was <5% of the mean surface area for the other groups.
[a] Values are mean treated:control ratio ± SE. The number of separate experiments in which myocyte protein content was determined is indicated in parentheses.
[b] $p < 0.001$ vs. control.

Nonmyocyte-derived growth factor appears to be a 45–50-kDa protein

Several 0.75 M NaCl fractions from heparin-Sepharose chromatography (representing 1 l of NMC-CM) were combined, concentrated to 100 μl, and loaded onto a gel filtration Superose-12 column. As shown in Figure 6, peak growth-promoting activity was found in fraction 34, corresponding to a molecular weight of 45–50 kDa, as determined by running standards of known molecular size on the sizing column. An aliquot of this fraction showed a major band of 45–50 kDa by silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and denaturing conditions. We will tentatively refer to this protein with growth-promoting activity for cardiac myocytes as the nonmyocyte-derived growth factor (NMDGF).

NMDGF may be distinct from other known growth factors

Cardiac tissue has been shown recently to contain several known peptide growth factors, including aFGF, bFGF, PDGF, TNF, and TGFβ_1 (Friedman *et al.*, 1988; Casscells *et al.*, 1989, 1990; Eghbali, 1989; Kardami and Fandrich,

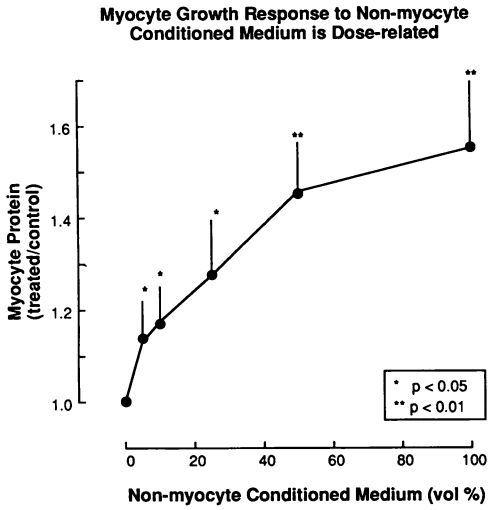


***Figure 3.* Myocyte hypertrophic growth response to NMC-CM is dose related.** Conditioned medium from nonmyocyte cultures was collected over 3 d, mixed with fresh MEM/TI in varying proportions (5–100% vol/vol), and added to myocyte cultures. Myocyte protein content was quantified after 2 d and compared with control cultures maintained in fresh MEM/TI without conditioned medium. Each point is the mean treated:control ratio for protein content ± SE for three culture preparations, each treated with a separate batch of conditioned medium. There were three dishes of cultured myocytes in each treated and control group of each experiment. *$p < 0.05$, **$p < 0.01$ vs. control.

1989; Weiner and Swain, 1989). The effects of these growth factors on myocyte size in primary culture were compared with that of the 0.75 M heparin-Sepharose fraction (Table 2 and Figure 7).

aFGF, bFGF, PDGF, TNFα, and TGFβ_1 each increased myocyte protein content in a dose-related manner, with approximate EC_{50}s (ng/ml) of 100, 5, 2, 5, and 2, respectively (Table 2 and Figure 7). However, the absolute magnitude of the myocyte size increase with the most potent of these growth factors (TNFα) was less than that observed with NMC-CM or affinity-purified and concentrated 0.75 M NaCl fraction (Figure 7). The nonconcentrated 0.75 M fraction at 50 μl/ml contained <1 ng protein but increased myocyte size similar to 1 μg TNFα (Table 2).

Under the same conditions used for heparin-Sepharose fractionation of NMC-CM, aFGF, bFGF, PDGF, and TGFβ_1 bound to heparin-Sepharose, whereas TNFα did not bind (Figure 8 and Table 2). aFGF, bFGF, and TGFβ_1 eluted from heparin-Sepharose at 1 M NaCl or higher, whereas the major activity in NMC-CM eluted at 0.75 M NaCl (Figure 8 and Table 2). PDGF also eluted at 0.75 M NaCl (Figure 8 and Table 2).

In an effort to further distinguish from PDGF the factor(s) present in NMC-CM and eluting with 0.75 M NaCl, 0.75 M fractions were coincubated with antibody to PDGF before testing. At an antibody concentration (100 μg/ml) adequate for neutralization of up to 10 ng/ml of all dimeric forms of human, bovine, and rodent PDGF, this antibody neutralized PDGF-stimulated ^{14}C-phenylalanine incorporation into myocytes but failed to inhibit that induced by the 0.75 M fraction (Table 3). Likewise, antibodies directed against aFGF, bFGF, TGFβ_1, and TNFα were tested. None of these antibodies neutralized the growth-promoting activity of NMDGF (1 experiment each, data not shown).

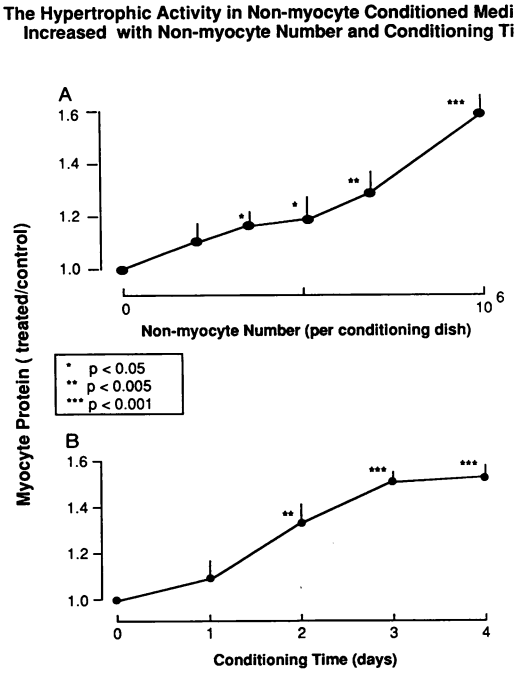


***Figure 4.* Growth-promoting activity in NMC-CM increases with nonmyocyte number and conditioning time.** Nonmyocyte cultures were established and maintained in serum-free MEM/TI as described in Materials and methods. Conditioned medium was collected, mixed with fresh MEM/TI (50% vol/vol), and added to myocyte cultures. Myocyte protein content was determined after 2 d in comparison with control cultures maintained in fresh MEM/TI without conditioned medium. Each point is the mean treated:control ratio for myocyte protein content ± SE for three dishes. (A) The number of nonmyocytes seeded into the dishes used for preparation of conditioned medium was varied. Conditioned medium was collected after 3 d of incubation. Nonmyocyte numbers were determined at the time of collection of conditioned medium and ranged from 2.5×10^5 to 1×10^6 per 100-mm dish. (B) Conditioned medium was collected over 1–4 d of incubation after the serum-free washing steps. The nonmyocyte cultures contained 6.8×10^5 cells per 100-mm dish. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ vs. control.

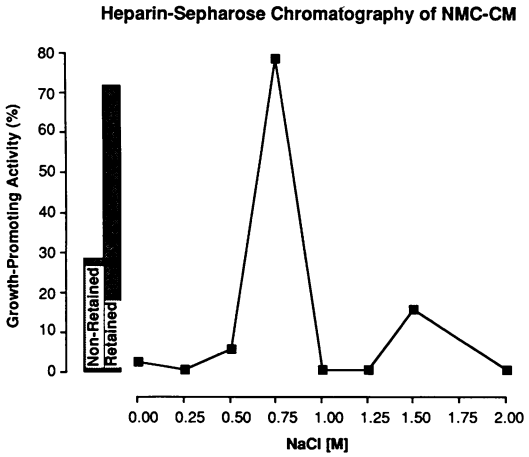


Figure 5. **Heparin-Sepharose chromatography of NMC-CM.** NMC-CM was analyzed by heparin-Sepharose chromatography. Aliquots of the eluants containing various concentrations of NaCl in 10 mM Tris (pH 7.5) were added to myocyte cultures, and myocyte protein content was determined after 2 d in comparison with controls treated with the appropriate concentration of NaCl. The percent retained was calculated by comparing the growth-promoting activity (^{14}C-phenylalanine incorporation into myocyte protein) of flow-through with an aliquot of the material initially applied to the column. Each point is the mean treated:control ratio for protein content in one experiment. The same pattern of heparin binding and NaCl elution was seen in five additional experiments.

Stimulation of phosphatidylinositol turnover

PDGF and TGFβ_1 have been shown to stimulate phosphatidylinositol turnover in some cells. Therefore, we examined the effects of 0.75 M heparin-Sepharose fractions of NMC-CM on phosphatidylinositol hydrolysis (PI), another characteristic that could potentially distinguish it from these other known factors. Incubation of myocytes with both PDGF (10 ng/ml, n = 3) and TGFβ_1 (2.5-10 ng/ml, n = 5) stimulated an increase in ^{3}H-inositol phosphate production (1.28 ± 0.06 and 1.10 ± 0.03-fold vs. control, mean ± SE for PDGF and TGFβ_1, respectively, $p < 0.05$). In contrast, treatment of myocytes with 0.75 M heparin-Sepharose fractions did not change ^{3}H-inositol phosphate production (1.00 ± 0.01-fold vs. control, n = 3, p = NS).

Cell specificity of NMC-CM production

Three other nonmuscle cell types were tested for their ability to produce myocyte growth-promoting activity. Conditioned media were prepared from cultures of the RAT-1 cell line, chick embryo fibroblasts, and COS-7 cells, using the same procedures as for the cardiac nonmyocytes. Conditioned media from these other cell types had no effect on cardiac myocyte size (data not shown, 1 experiment with medium from each cell type).

Cell specificity of NMDGF action

To test the cell specificity of NMDGF, 0.75 M heparin-Sepharose fractions of NMC-CM were tested for their ability to stimulate DNA synthesis in other types of cells. Smooth muscle cell

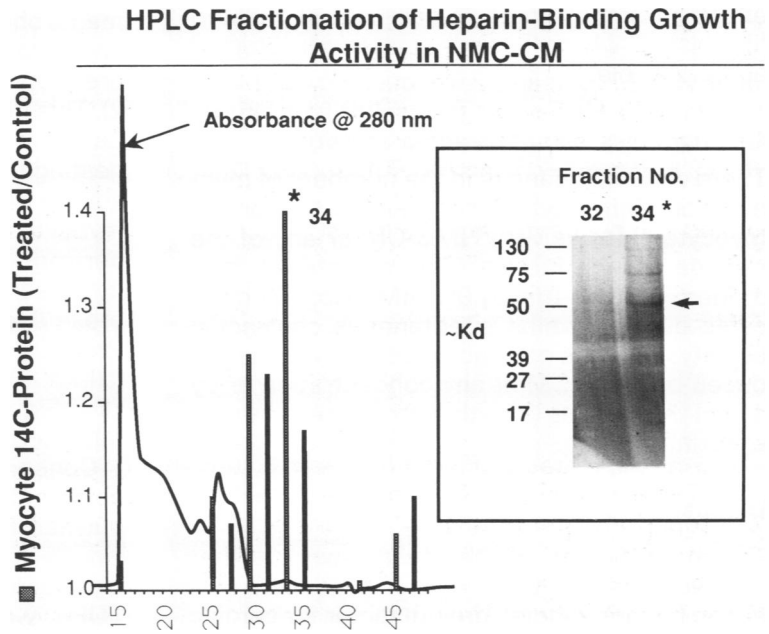


Figure 6. **Size-exclusion HPLC and SDS-PAGE of 0.75 M fractions of NMC-CM.** 0.75 M NaCl fractions from heparin-Sepharose chromatography of 1 l of NMC-CM were combined, concentrated, and applied to a Superose-12 sizing column and eluted with 10 mM Tris, pH 7.5, 0.75 M NaCl at 0.5 ml/min. Fractions (0.5 ml) were collected and assayed for growth-promoting activity on cardiac myocytes. The fraction with the most activity (fraction 34) was then analyzed by SDS-PAGE. A major band is seen at 45–50 kDa after silver staining. Fraction 32, which contained less activity than fraction 34, has a faint band at 45–50 kDa but no bands at 130 and 75 kDa. Similar results have been obtained in two additional experiments.

Table 2. Growth-promoting effects and heparin-sepharose chromatography of NMC-CM and NMDGF compared with aFGF, bFGF, PDGF, TNFα, and TGFβ_1

Growth factor (final concentration)	Myocyte protein (fold-increase)	Heparin-binding	Heparin-Sepharose elution (NaCl, M)
aFGF (1 μg/ml)	1.24 ± 0.04 (4)[a]	Yes	1–2
bFGF (100 ng/ml)	1.13 ± 0.01 (6)[c]	Yes	1–2
PDGF (20 ng/ml)	1.25 ± 0.03 (11)[a]	Yes	0.75
TNFα (1 μg/ml)	1.31 ± 0.05 (5)[b]	No	—
TGFβ_1 (20 ng/ml)	1.24 ± 0.01 (3)[c]	Yes	1–2
NMC-CM (50% vol/vol)	1.42 ± 0.04 (26)[b]	Yes	0.75 (80% of total activity)
NMDGF (0.75 M NaCl fraction, 50 μl/ml)	1.30 ± 0.02 (25)[c]	Yes	0.75

Cultured cardiac myocytes were treated on culture day 1 with a concentration of each growth factor producing a maximum increase in myocyte size. After 2–3 d, myocyte protein content was determined as described in Materials and methods. Values for myocyte protein are means ±SE treated/control ratio, with the number of culture preparations in parentheses (cf Figure 7). In separate experiments, the peptide growth factors were added to culture medium and analyzed by heparin-Sepharose chromatography, using exactly the same protocol employed for NMC-CM. For factors that bound to heparin-Sepharose, the NaCl concentration which eluted myocyte growth factor activity is indicated (cf Figures 5 and 8).
[a] $p < 0.01$, [b] $p < 0.005$, [c] $p < 0.001$ vs. vehicle-treated control myocytes.

DNA synthesis was stimulated potently by 5% calf serum (>20-fold increase in ^{3}H-thymidine incorporation), but the 0.75 M fraction increased DNA synthesis only 5% as much as did serum (5.0 ± 0.05% of the effect of calf serum, mean ± SE, n = 3). In contrast to the results with smooth muscle cells, treatment of NIH 3T3 cells and cardiac nonmyocytes with 0.75 M heparin-Sepharose fractions of cardiac NMC-CM did stimulate ^{3}H-thymidine incorporation (104 ± 9% and 55 ± 3% of the effect of calf serum, mean ± SE, for 3T3 cells and cardiac nonmyocytes, respectively, n = 3 each). Thus, NMDGF had no effect on smooth muscle cells but stimulated fibroblast DNA synthesis, suggesting a potential autocrine role.

Cell numbers and contractile activity

There were no changes in the numbers of myocytes or nonmyocytes after treatment of cardiac myocyte cultures with NMC-CM or any of the peptide growth factors (data not shown, bromodeoxyuridine [BrdU] 0.1 mM). Control myocytes did not exhibit spontaneous contractile activity. Myocyte contractile activity was not induced by NMC-CM at any concentration or by any of the peptide growth factors used in these experiments.

Discussion

The major finding of the present work is that cardiac nonmyocytes in culture produce a paracrine heparin-binding growth factor for cardiac myocytes. This factor also acts in an autocrine fashion to stimulate fibroblast DNA synthesis. This growth factor appears not to be one of the other polypeptide growth factors that have been found previously in heart tissue, namely aFGF, bFGF, PDGF, TGFβ_1, or TNFα. We have tentatively named this factor NMDGF pending determination of amino acid sequence and analysis of its relationship to other known growth factors and growth factor families.

Our first observations indicating a potentially important nonmyocyte/myocyte interaction on myocyte growth in culture came from experiments showing hypertrophy of myocytes grown under conditions of increased cell density (Figure 1) and with cocultured nonmyocytes (Figure 2). This myocyte growth-promoting effect could be reproduced by NMC-CM (Figure 3), suggesting that the nonmyocytes were indeed the source of a factor or factors that acted on the myocytes in a paracrine fashion to cause hypertrophy. Most of the growth-promoting activity present in NMC-CM bound to heparin-Sepharose and eluted with 0.75 M NaCl (Figure 5). Preliminary evidence suggested that this factor (NMDGF) has a molecular weight of ~45–50 kDa (Figure 6), but this point has not been established with certainty.

Concerning the identity of NMDGF, the available evidence suggests that it can be distinguished from those growth factors found previously in myocardium. Several of these growth factors, namely aFGF, bFGF, PDGF, TGFβ_1, and TNFα, were also found to stimulate myocyte

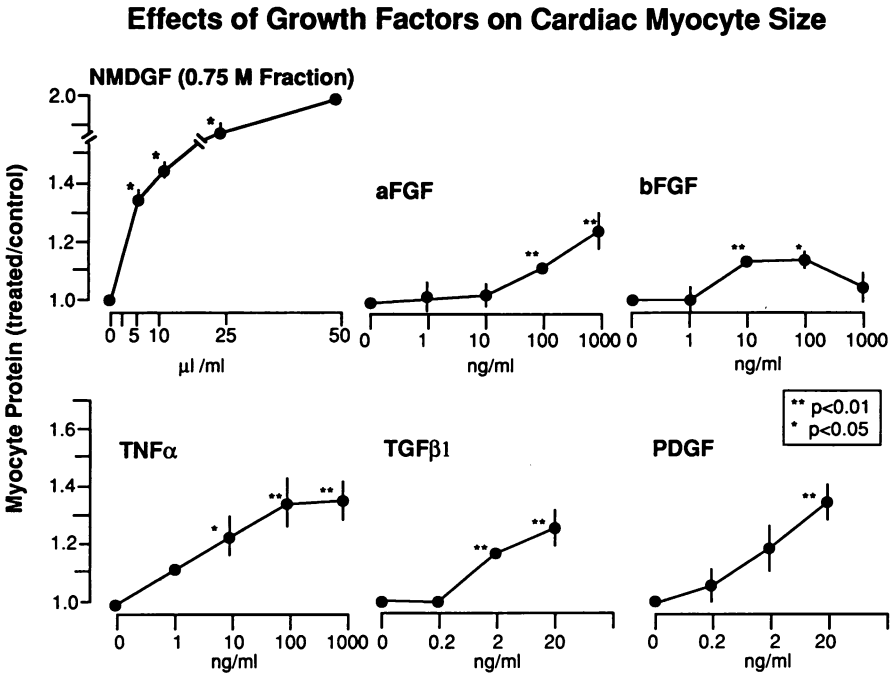


Figure 7. **Growth response of myocytes to the 0.75 M NaCl heparin-Sepharose fraction compared with the hypertrophic response to other peptide growth factors.** Varying concentrations of individual growth factors were added to myocyte cultures freshly renewed with 1 ml MEM/TI. ^{14}C-phenylalanine incorporation into trichloroacetic acid-insoluble, SDS-soluble material was determined after 2 d and compared with vehicle-treated control cells. Each point represents the mean treated:control ratio ± SE for protein content in three separate experiments. For the 0.75 M heparin-Sepharose fraction, the 0.75 M fraction was concentrated fivefold with a microconcentrator and aliquots were added to fresh MEM/TI (n = 2). *$p < 0.05$, **$p < 0.01$ vs. control.

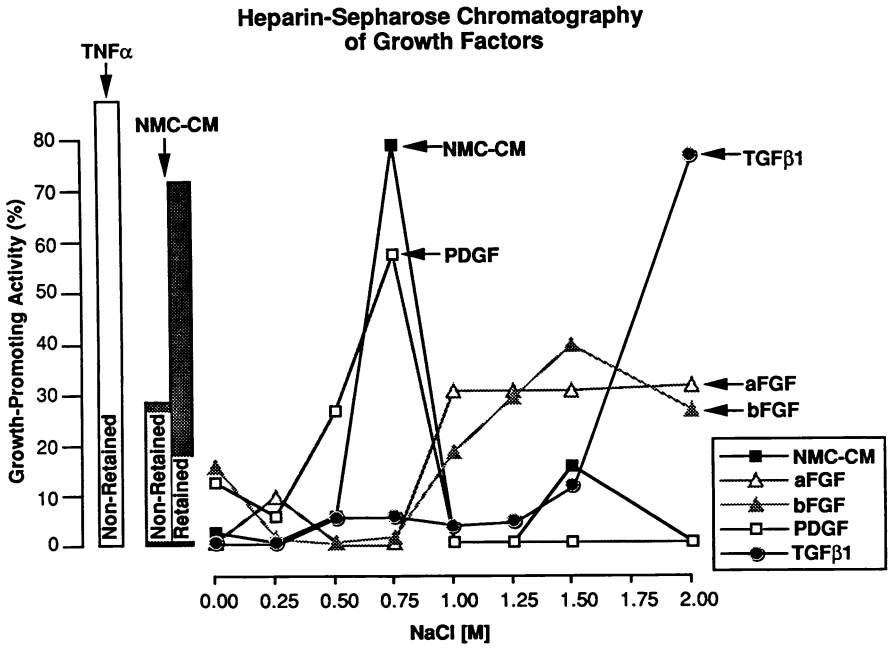


Figure 8. **Heparin-Sepharose chromatography of other known peptide growth factors found in heart.** Growth factors were added to MEM/TI and subjected to heparin-Sepharose chromatography in the same manner as for NMC-CM. Aliquots of the eluants containing various concentrations of NaCl in 10 mM Tris (pH 7.5) were added to myocyte cultures, and myocyte protein content was determined after 2 d, in comparison with controls treated with the appropriate concentration of NaCl. Each point is the mean treated:control ratio for protein content in one experiment (3 culture dishes/NaCl fraction). The heparin-Sepharose profile of NMC-CM (Figure 5) is included for comparison. The elution patterns for PDGF (Shing *et al.*, 1984; Besner *et al.*, 1990) and for aFGF and bFGF (Speir *et al.*, 1988; Weiner and Swain, 1989; Casscells *et al.*, 1990) are the same as those described by others. The profile shown for TGFβ1 was seen in two additional experiments.

Table 3. Anti-PDGF antibody neutralizes PDGF-stimulated myocyte growth but not NMDGF-stimulated growth

Growth factor	Anti-PDGF antibody	Myocyte protein (fold-increase)
PDGF	No	1.12 ± 0.02[a]
PDGF	Yes	1.04 ± 0.01
NMDGF	No	1.27 ± 0.04[a]
NMDGF	Yes	1.26 ± 0.08[a]

Myocyte protein content was determined after 3 d treatment with PDGF (10 ng/ml) or NMDGF (the heparin-binding growth factor eluting at 0.75 M NaCl, 50 μl/ml) in the absence or presence of neutralizing polyclonal antibody to PDGF. Based on the data with concentrated 0.75 M fraction in Figure 7, 50 μl/ml of nonconcentrated 0.75 M fraction produces a response ~ 40% of maximum. The anti-PDGF-AB antibody was used at 100 μg/ml, a dose adequate for neutralization of 10 μg/ml of all dimeric forms of PDGF. Values for the fold-increase in myocyte protein are the means ± SE for three experiments.

[a] $p < 0.05$ vs. vehicle-treated control cells.

growth in culture (Figure 7). Of these five, bFGF, TGFβ_1, and TNFα have been localized to the nonmyocytes (Friedman *et al.*, 1988; Eghbali, 1989; Kardami and Fandrich, 1989; Weiner and Swain, 1989). aFGF mRNA has been found in myocyte cultures (Weiner and Swain, 1989). PDGF mRNA has not been localized to either cell-type specifically, only being detected in whole heart RNA (Sarzani *et al.*, 1991). Although TNFα has been reported to be produced by cultured heart nonmyocytes in response to lipopolysaccharide administration (Friedman *et al.*, 1988), the fact that it does not bind to heparin-Sepharose eliminates it as a candidate for the nonmyocyte-derived factor described here (Figure 8). Although aFGF, bFGF, and TGFβ_1 are found in cardiac cells, their heparin-Sepharose elution profiles are distinct from NMDGF (Table 2 and Figure 8). PDGF, however, binds to and elutes from heparin-Sepharose in a manner similar to NMDGF (Table 2) (Shing *et al.*, 1984; Besner *et al.*, 1990). Therefore, it was critical to consider whether PDGF was the active factor in our NMC-CM. Evidence that PDGF is not the active factor in the 0.75 M NaCl fraction is fivefold: 1) in contrast to the factor in NMC-CM, PDGF is heat-insensitive (DiCorleto, 1984); 2) anti-PDGF antibody failed to neutralize the growth-promoting activity of the 0.75 M fraction (Table 3); 3) Growth-promoting 0.75 M fractions of NMC-CM did not stimulate PI hydrolysis, in contrast to the stimulation seen with both PDGF and TGFβ_1; 4) 0.75 M heparin-Sepharose fractions of NMC-CM did not stimulate smooth muscle cell DNA synthesis, whereas both PDGF and TGFβ_1 do so (Ross *et al.*, 1986; Battegay *et al.*, 1990; Hamet *et al.*, 1991); and 5) the putative 45–50 kDa molecular weight of NMDGF is larger than that of the previously mentioned growth factors, including PDGF (25 kDa).

About 70% of the growth-promoting activity in NMC-CM bound to heparin-Sepharose, and ~20% of the bound activity eluted with 1.5 M NaCl (Figure 5). The identity of the factor(s) in NMC-CM eluting from the heparin-Sepharose column with 1.5 M NaCl is unknown at present. Because 1–2 M NaCl is the salt concentration at which both bFGF and TGFβ_1 eluted, it is tempting to speculate that the 1.5 M fraction contains one or both of these factors. Both bFGF and TGFβ_1 mRNAs have been localized to cardiac nonmyocytes (Eghbali, 1989; Weiner and Swain, 1989). Further work will be necessary to determine the exact identity of the factor or factors in the 1.5 M NaCl fractions of NMC-CM. About 30% of the growth-promoting activity in NMC-CM did not bind to heparin-Sepharose. Because TNFα does not bind to heparin-Sepharose, it might be responsible for the 30% of the growth-promoting effect of NMC-CM found in the flow-through fraction of our columns.

Because serum can be a source of growth-promoting factors, it was important to establish that the growth-promoting activity in NMC-CM was cell-derived and not serum-derived. Several lines of evidence support this conclusion. 1) The growth response of myocytes to NMC-CM was a function of nonmyocyte number and conditioning time (Figure 4). 2) The lack of growth response to mock-conditioned medium or to conditioned medium from equivalent numbers of myocytes indicated that nonmyocytes were required for myocyte growth-promoting activity. 3) In coculture experiments, myocyte size was a function of nonmyocyte number (Figure 2). All of these cultures were treated in exactly the same way, the only variable being number of nonmyocytes added. 4) Extensive washing of nonmyocyte cultures with serum-free medium before collection of conditioned medium made it unlikely that serum would be retained in sufficient concentration to cause growth (Simpson *et al.*, 1982). Serum treatment also causes beating of cultured cardiac myocytes (Simpson *et al.*, 1982), which was not observed in response to NMC-CM. 5) Finally, inclusion of cycloheximide in nonmyocyte cultures during preparation of conditioned medium reduced

production of growth-promoting activity and total nonmyocyte protein synthesis by similar amounts. All of these points indicate that the muscle cell growth response to NMC-CM is not due to a retained serum component(s).

Our observations that cardiac nonmyocytes can influence cardiac myocyte growth may have important implications in vivo. Nonmyocyte numbers are increased in hypertrophic and dilated cardiomyopathies and in response to ischemic injury and infarction (Morkin and Ashford, 1968; Carroll *et al.,* 1989; Huysman *et al.,* 1989; Weber *et al.,* 1989; Chapman *et al.,* 1990). Additionally, under these pathological circumstances, the nonmyocytes produce increased amounts of interstitial collagen (types I, III, and IV), which adversely effect cardiac function and can result in additional injury (Carroll *et al.,* 1989; Brilla *et al.,* 1991). The exact mechanism through which nonmyocyte proliferation and collagen production are induced in these situations is unclear but may involve some of the growth factors produced by the nonmyocytes themselves through autocrine mechanisms.

In addition to the paracrine induction of myocyte growth demonstrated here, recent evidence suggests that nonmyocyte/myocyte interactions may also result in alterations in myocyte gene expression. Administration of aFGF, bFGF, and TGFβ_1 to neonatal rat cardiac myocytes in culture results in the induction of fetal/neonatal contractile protein gene transcription, simulating that seen in pressure-load hypertrophy in vivo (Parker *et al.,* 1990a,b). In support of these observations, preliminary evidence in our laboratory suggests that NMC-CM can induce expression of transfected skeletal α-actin promoter constructions (Karns, Long, and Simpson, unpublished data). An active role of cardiac nonmyocytes in cardiac morphogenesis is also suggested by reports that members of the TGFβ family may be involved in embryonic atrioventricular valve formation (Potts and Runyan, 1989; Potts *et al.,* 1991). Additionally, there may be direct cell–cell interactions that are not addressed here. For example, chick heart fibroblasts have been shown to physically move cardiac muscle cells, organizing them into multicellular beating units in mixed cultures, a phenomenon involving direct cell–cell contact (Gross, 1982).

The increase in myocyte size with increasing cell culture density (Figure 1) could have been explained by the paracrine factor(s) described here, and there was no evidence for autocrine growth factors in medium conditioned by myocytes (Table 1). On the other hand, aFGF might be an autocrine myocyte growth factor (Parker *et al.,* 1990a,b; Weiner and Swain, 1989) (Figure 7).

In summary, cardiac nonmyocytes, which comprise ~70% of the total cell numbers in mature myocardium, stimulate growth of cardiac muscle cells in culture, at least in part via the production of a novel heparin-binding growth factor. The nonmyocytes may modulate myocyte growth, development, and response to injury in vivo by production of one or more of the growth factors studied here. Furthermore, the increases in nonmyocyte numbers in pathological conditions may involve autocrine mechanisms suggested by this work.

Materials and methods

Materials

Bovine aFGF, bFGF, and polyclonal antibodies against these growth factors were gifts from D. Gospodarowicz, the University of California, San Francisco. Human platelet PDGF (AA, AB, and BB) was either purchased from Sigma (St. Louis, MO) or obtained from J. Escobedo and L.T. Williams, the University of California, San Francisco. Neutralizing polyclonal antibody to human PDGF-AB was obtained from UBI Biochemicals (Lake Placid, MI). Human recombinant TNFα was donated by Genentech (South San Francisco, CA). Neutralizing polyclonal antibody to rat TNFα was a gift from C. Grunfeld, University of California, San Francisco, and the San Francisco VA Medical Center. Porcine platelet TGFβ_1 and neutralizing polyclonal antibody to porcine TGFβ_1 were obtained from R & D Systems (Minneapolis, MN). MEM with Hank's salts was obtained from the University of California, San Francisco Cell Culture Facility. Calf serum was obtained from Hyclone (Logan, UT). Insulin was obtained from Lilly (Indianapolis, IN). Monoclonal antibody to striated muscle myosin (MF-20) (Bader *et al.,* 1982) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). Polyclonal antibody to smooth muscle myosin was the gift of J. Woodcock and J. Mitchell, University of Vermont, Burlington. Monoclonal antibody to smooth muscle actin was obtained from Sigma. Polyclonal antibody to Factor VIII was obtained from Dakopatts (Glostrup, Denmark). Acetylated low-density lipoprotein (LDL) was a gift from R. Pitas, University of California, San Francisco, and the San Francisco General Hospital.

Cell culture

Myocyte cultures. Cardiac myocytes were isolated from day-old rat hearts and maintained at low density in serum-free MEM supplemented with 10 μg/ml each of transferrin and insulin, 0.1 mM BrdU, and 1 mg/ml of bovine serum albumen (MEM/TIB-BSA). After dissociation of the hearts with trypsin, cells were preplated for 30 min in MEM/5% calf serum to reduce nonmyocyte numbers (Simpson and Savion, 1982). Myocyte yield was ~5×10^6 per heart. Final cell density was ~150 per mm^2 in 35-mm culture dishes (Falcon, Lincoln Park, NJ). Nonmyocytes in the myocyte cultures were limited to ~10% or less of total cell number by inclusion of 0.1 mM BrdU in the medium for the first 3 d of culture (Simpson and Savion, 1982). The myocytes were

used for experiments on culture day 1 or 4. Results were the same with cultures used on either day.

To examine the effect of cell density in the myocyte cultures on myocyte size, the seeding density was varied such that the final cell density covered the range from 10 to 400 cells/mm^2.

For coculture experiments, myocytes obtained after preplating were mixed with varying numbers of nonmyocytes, plated as usual in MEM/5% calf serum with 0.1 mM BrdU to prevent nonmyocyte proliferation, and allowed to attach overnight. The cocultures were maintained in MEM/TIB-BSA for up to 5 d.

To prepare medium conditioned by myocytes, the myocytes were plated into 100-mm culture dishes (Falcon) in MEM/5% calf serum and 0.1 mM BrdU. Final cell density was ~180 per mm^2 (1×10^6 cells per dish). Conditioned medium was prepared as described below for trypsinized nonmyocytes.

Cardiac nonmyocyte cultures. Homogeneous nonmyocyte cultures were prepared from the preplates which contained predominantly (>95%) attached nonmyocytes. The 100-mm preplates were scraped with a sterile plastic scraper 1–2 h after preplating, and the cells were allowed to reattach overnight in MEM/5% calf serum. The few myocytes in the preplates did not survive this procedure. Medium was renewed and the nonmyocytes were maintained without medium changes in MEM/5% calf serum. When the cells had proliferated to confluence, they were removed by trypsinization (1 mg/ml in phosphate-buffered saline at 37°C for 15 min) and used for coculture with muscle cells.

For production of NMC-CM, preplates were scraped on day 1 and the nonmyocytes were collected by centrifugation ($500 \times g$). The nonmyocytes were resuspended in MEM/5% calf serum, plated onto 100-mm dishes, and maintained in this medium (renewed every 3 d) until confluent (7–10 d).

Other nonmyocyte cultures. RAT-1 fibroblasts and COS-7 cells were obtained from the University of California, San Francisco, Cell Culture Facility. Primary chick embryo fibroblasts were obtained from S. Hawkes, the University of California, San Francisco. These cells were cultured in the same manner as the rat cardiac nonmyocytes and were used to test for the cardiac nonmyocyte specificity of conditioned medium production.

For DNA synthesis assays, NIH 3T3 and primary cultures of canine tracheal smooth muscle cells were obtained from the University of California, San Francisco, Cell Culture Facility and J. Brown, San Francisco VA Medical Center, respectively. These cells were grown to confluence in MEM/5% calf serum before testing.

Identity of the cardiac nonmyocytes

Preliminary studies with immunofluorescence, performed as described previously (Mochly-Rosen *et al.*, 1990) and with vital staining using acetylated LDL, indicated that the neonatal rat heart nonmyocytes in primary culture were probably fibroblasts. The nonmyocytes did not react with antibody to striated or smooth muscle myosin heavy chain. They did stain intensely with anti-smooth muscle actin, showing a stress fiber appearance of the staining pattern. The nonmyocytes did not take up acetylated LDL and did not stain with antibody to factor VIII, suggesting that they were not endothelial in origin. Electron microscopic studies indicate that these cells contain large amounts of rough endoplasmic reticulum and intermediate-type microfilaments (Simpson and Savion, 1982).

Production of NMC-CM

Once the nonmyocytes reached confluence, MEM/5% calf serum was removed and the cells were washed extensively with MEM/TI. After overnight incubation with 10 ml MEM/TI per dish, fresh MEM/TI was added (10 ml/dish). NMC-CM was then collected after 3 d of additional incubation. To determine whether growth-promoting activity increased with conditioning time, NMC-CM was collected after incubation times of 1–4 d.

In most experiments, the final nonmyocyte number was ~1×10^6 per 100-mm dish (180 per mm^2). To study whether growth-promoting activity was related to nonmyocyte number, the number of nonmyocytes was varied between 2.5 $\times 10^5$ and 1×10^6 per 100-mm dish. To ask if the growth-promoting activity of conditioned medium required nonmyocyte protein synthesis, conditioned medium was collected from nonmyocyte cultures treated with or without cycloheximide (2 μM, 0.6 μg/ml).

NMC-CM was either used immediately or frozen at −72°C without loss of activity for up to 6 mo after collection. For testing, NMC-CM was mixed with fresh MEM/TI (50% vol/vol) and added to the myocyte cultures. Myocyte size (see below) was determined after 2–3 d in the presence of NMC-CM. To determine the dose dependency of the growth response to NMC-CM, varying proportions in fresh MEM/TI were used (5–100% by volume).

Three different control media were used in comparison with NMC-CM: 1) medium mock-conditioned in dishes without cells; 2) medium conditioned by muscle cells maintained in 100-mm dishes; and 3) fresh MEM/TI. The first two media were prepared exactly the same as NMC-CM.

NMC-CM sensitivity to heat, protease digestion, and reduction with DTT (50 mM) was investigated. NMC-CM and fresh MEM/TI control were heat-treated (95°C, 20 min) or treated with trypsin (0.1 mg/ml, 1 h, 37°C, followed by addition of 0.5 mg/ml soybean trypsin inhibitor) before determination of their effect on muscle cell size. For DTT experiments, NMC-CM was treated with 50 mM DTT for 30 min at 37°C followed by passage over heparin-Sepharose as described below.

Cardiac myocyte number and size

Cell numbers and myocyte size were determined after 2–3 d treatment with NMC-CM or other growth factors, in comparison with cells treated with control media or growth factor diluents. Cell numbers and the proportions of nonmyocytes in the myocyte cultures were determined by counting all cells in randomly-selected microscopic fields (Simpson and Savion, 1982; Simpson *et al.*, 1982). Cardiac myocyte size was quantified using two assays, surface area and radiolabeled protein content, both of which have been described and validated previously as measures of cultured myocyte hypertrophy in response to growth factors (Simpson and Savion, 1982; Simpson *et al.*, 1982). The surface area of attached myocytes was determined by image analysis (Simpson *et al.*, 1982). All myocytes in randomly selected microscopic fields were measured and ≥75–100 cells were quantified in each group. Myocyte protein content was estimated by continuous labeling of cell protein with ^{14}C-phenylalanine during treatment with NMC-CM, heparin-Sepharose fractions, or peptide growth factors. There were at least three myocyte culture dishes in each treated and control group. Increased radiolabeled protein accumulation was not due to cell proliferation because cell numbers did not change with any of the treatments described. The effect of antibodies to aFGF, bFGF, PDGF, TNFα, and TGFβ_1 on the myocyte hypertrophic response to NMC-CM or 0.75 M hep-

arin-Sepharose fraction were individually determined by incubation with antibody at 37°C for 30 min before addition of the growth-promoting agents to the myocyte cultures.

Myocyte contractile activity

Control myocytes at low density in serum-free medium are quiescent and do not exhibit spontaneous contractile activity (Simpson, 1985). The development of spontaneous contractility was assessed by microscopic examination of the cultures at 37°C (Simpson, 1985).

DNA synthesis

DNA synthesis of nonmyocytes, NIH 3T3, and canine tracheal smooth muscle cells was assayed by measuring the incorporation of ^{3}H-thymidine into DNA, using an adaptation of the method of Shing *et al.* (1984). In brief, cells were plated in 24-well plates and grown over a period of 5–7 d in MEM/5% calf serum until confluent. MEM/5% calf serum was replaced with MEM/TI-BSA for 24 h, after which growth factor samples (or their appropriate controls) were added to fresh MEM/TI-BSA. After 1 d at 37°C, ^{3}H-thymidine (10 μCi/ml) was added. One day later, the dishes were washed with phosphate-buffered saline and trichloroacetic acid-insoluble, SDS-soluble material was collected and counted. DNA synthesis stimulated by 5% calf serum was used as the standard (100% stimulation). The effect of 0.75 M heparin-Sepharose fractions were then expressed as the percent stimulation of cells concomitantly treated with 5% calf serum.

Chromatography

Heparin-Sepharose chromatography. NMC-CM (50 ml) was first concentrated 10-fold by ultrafiltration (Pharmacia Filtron 8000 MW cutoff filter, Pharmacia, Piscataway, NJ, prewashed twice with 10 ml water). One-tenth volume of 100 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5) was added, and the concentrated conditioned medium (5 ml) was poured onto a 0.75 × 1-cm column of heparin-Sepharose (heparin-Sepharose CL-6B, Pharmacia), pre-equilibrated and washed extensively with 10 mM Tris, pH 7.5. After addition of conditioned medium, the column was washed with 5 ml of 10 mM Tris (pH 7.5) and batch-eluted with 4 to 5 column volumes (2 ml) of a NaCl step gradient (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 M NaCl in 10 mM Tris, pH 7.5). All columns were run at 4°C. After sterilization by filtration (Millipore Millex GV 0.22 μm, Millipore, Bedford, MA), aliquots of each fraction were assayed for their effect on myocyte size (50 μl in 1 ml of MEM/TI). Control myocytes were treated with 10 mM Tris (pH 7.5) with the same NaCl concentration as the tested fraction. Similar heparin-Sepharose profiles were obtained using prepackaged columns (Econopac heparin, BioRad, Richmond, CA) and applied NMC-CM volumes of up to 300 ml. For heparin-Sepharose chromatography of other growth factors (aFGF, bFGF, PDGF, TNFα, and TGFβ), each factor was mixed individually with MEM/TI containing 10 mM Tris (pH 7.5), loaded onto the heparin-Sepharose column, eluted, and assayed in exactly the same manner as described above for NMC-CM.

Gel filtration chromatography. The 0.75 M NaCl fractions from heparin-Sepharose chromatography (representing 1 l of NMC-CM) were pooled, concentrated with a 10 000 MW cut-off Centricon microconcentrator (Amicon, Danvers, CA) and loaded onto a size-exclusion Superose-12 (Pharmacia) high-pressure liquid chromatography (HPLC) column. The column was eluted with 0.75 M NaCl in 10 mM Tris, pH 7.5 (0.5 ml/min), and 0.5-ml fractions were collected and assayed for activity on cultured myocytes. Active HPLC fractions could be stored at −72°C without loss of activity for subsequent gel electrophoresis (SDS-PAGE) and testing.

PAGE

Fractions from gel filtration HPLC having growth-promoting activity for cardiac myocytes were analyzed by reducing, denaturing SDS-PAGE (Laemmli, 1970) followed by silver staining (BioRad).

Phosphatidylinositol hydrolysis

Phosphatidylinositol hydrolysis was determined as described previously (Karliner *et al.*, 1990). In brief, phosphatidylinositides were radiolabeled by incubating cardiac myocytes with myo-[2-^{3}H] inositol, 1 μCi/ml, 3.9 Ci/mmol (Amersham, Arlington Heights, IL) at 37°C for 24 h. Cells were washed three times with 2 ml MEM/TIB with 20 mM LiCl and equilibrated at 37°C for 10 min in 1 ml MEM/TI with LiCl. After addition of 0.75-M heparin-Sepharose fraction or growth factor, cells were incubated for 1 h at 37°C. Total cellular ^{3}H-inositol phosphates were then extracted with 10% trichloroacetic acid, bound to an anion exchange column (Dowex AG1-X8 100-200 mesh, BioRad), and eluted with 1 M ammonium formate/100 mM formic acid after extensive washing with water and 60 mM sodium formate/5 mM borax. Total ^{3}H-inositol phosphates were counted in Hionic fluor (Packard).

Statistics

Results are given as means ± SE. Mean values for two groups were compared using Student's t test, and analysis of variance with the Student-Newman-Keuls test was used for more than two groups. Treated:control ratios were tested for their deviation from unity by calculation of confidence limits (Snedecor and Cochran, 1967).

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