Cardiac fibroblasts influence cardiomyocyte phenotype in vitro

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1Department of Pathology and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Shadyside Hospital, Pittsburgh; and Departments of 2Cardiothoracic Surgery, 3Genomic Sciences, 4Pathology, and 5Pediatrics, Drexel University School of Medicine, Allegheny General Hospital, Pittsburgh, Pennsylvania

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LaFramboise WA, Scalise D, Stoodle P, Graner SR, Guthrie RD, Magovern JA, Becich MJ. Cardiac fibroblasts influence cardiomyocyte phenotype in vitro. Am J Physiol Cell Physiol 292: C1799–C1808, 2007. First published January 17, 2007; doi:10.1152/ajpcell.00166.2006.—Cardiac fibroblasts impact myocardial development and remodeling through intercellular contact with cardiomyocytes, but less is known about noncontact, profibrotic signals whereby fibroblasts alter cardiomyocyte behavior. Fibroblasts and cardiomyocytes were harvested from newborn rat ventricles and separated by serial digestion and gradient centrifugation. Cardiomyocytes were cultured in 1) standard medium, 2) standard medium diluted 1:1 with PBS, or 3) standard medium diluted 1:1 with medium conditioned ≥72 h by cardiac fibroblasts. Serum concentrations were held constant under all media conditions, and complete medium exchanges were performed daily. Cardiomyocytes began contracting within 24 h at clonal or mass densities with <5% of cells expressing vimentin. Immunocytochemical analysis revealed progressive expression of α-smooth muscle actin in cardiomyocytes after 24 h in all conditions. Only cardiomyocytes in fibroblast-conditioned medium stopped contracting by 72 h. There was a significant, sustained increase in vimentin expression specific to these cultures (means ± SD: conditioned 46.3 ± 6.0 vs. control 5.3 ± 2.9%, P < 0.00025) typically with cardiac myosin heavy chain coexpression. Proteomics assays revealed 10 cytokines (VEGF, GRO/KC, monocyte chemotactic protein-1, IL-6, IL-10, IL-12p70, IL-17, and tumor necrosis factor-α) at or below detection levels in unconditioned medium that were significantly elevated in fibroblast-conditioned medium. Latent transforming growth factor-β and RANTES were present in unconditioned medium but rose to higher levels in conditioned medium. Only granulocyte-macrophage colony-stimulating factor was present above threshold levels in standard medium but decreased with fibroblast conditioning. These data indicated that under the influence of fibroblast-conditioned medium, cardiomyocytes exhibited marked hypertrophy, diminished contractile capacity, and phenotype plasticity distinct from the dedifferentiation program present under standard culture conditions.

proteomics; myosin heavy chain; vimentin; myofibroblast; primary culture; dedifferentiation; plasticity

Recent discoveries that postnatal cardiomyocytes exhibit limited proliferative capacity and the identification of a cardiomyocyte progenitor cell source in the heart have led to reconsideration of mechanisms underlying the myocardial injury response (3, 30, 51). Studies have demonstrated successful myocardial regeneration under unique conditions, e.g., in MRL mice that manifest intrinsic high cardiomyocyte mitotic indices and in transgenic mice with targeted expression of cyclin D2 (23, 33, 34). In these exceptional cases, as in the case of fetal heart injury, restorative growth associated with cardiomyocyte replication serves to inhibit fibroblast-mediated remodeling (5, 44).

In vitro studies have established that signals induced by myocardial injury affect fibroblasts and cardiomyocytes and may regulate the degree of fibrosis and hypertrophy associated with the recovery process (16, 17, 20, 32). For example, complex cytokine-mediated interactions dissected in vitro revealed activation of mitosis in cardiomyocytes with concomitant suppression of fibroblast proliferation. These intercellular signals, e.g., interleukin (IL)-1β, IL-6, and transforming growth factor (TGF)-β, as well as various undefined soluble factors, may be of nonmyocardial, epicardial, or myocardial in vivo origin and act through paracrine or autocrine pathways (16, 17, 25, 32, 37, 41–43). Important cardiac fibroblast-cardiomyocyte interactions have been demonstrated in appositional cocultures involving direct membrane contact and/or cell fusion as well as through the use of noncontact cell culture paradigms to assess the role of soluble factors (12, 13, 17, 42).

The present study examined the effect of medium containing factors derived from cardiac fibroblasts on the behavior of highly purified, isolated ventricular cardiomyocytes. The findings demonstrated that cardiac fibroblasts induced changes in cardiomyocyte phenotype different from or in addition to the in vitro dedifferentiation process. These changes included alteration of myocyte structural and functional characteristics including hypertrophy, intracellular expression of vimentin, and reduction of chronotropic contractile activity. High-sensitivity protein analysis of cytokines and chemokines implicated in this response revealed 10 factors at negligible levels in standard medium but present at significantly higher levels in the conditioned medium including vascular endothelial growth factor (VEGF), growth-regulated α-protein (GRO/KC), monocyte chemotactic protein-1 (MCP-1), leptin, macrophage inflammatory protein-1α (MIP-1α), IL-6, IL-10, IL-12p70, and IL-17, and tumor necrosis factor (TNF)-α. Furthermore, two factors present in standard medium were significantly elevated

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after cardiac fibroblast conditioning including latent TGF-β and RANTES (regulated on activation of normal T cell expressed and secreted). Only one factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), fell significantly below concentrations obtained in standard medium as an effect of the conditioning process.

MATERIALS AND METHODS

Cardiac cell purification. Hearts were removed aseptically from neonatal (2–3 days old) Sprague-Dawley rats immediately after euthanasia with CO2. The study was performed according to the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996 revision), and procedures were performed at Zivic Laboratories (Zelienople, PA) under Institutional Animal Care and Use Committee approval (SVM03.00). The atria were removed and the ventricles placed in cold ADS buffer containing antibiotics [streptomycin and penicillin (50 U/ml; GIBCO BRL, Carlsbad CA); ampicillin (100 mg/ml; Sigma, St. Louis, MO)] and antimycotic amphotericin B (5 μg/ml; Mediatech, Herndon, VA). The ventricles (n = 20–25) were finely minced with iris scissors in ADS buffer to form a slurry. The minced tissue was resuspended in 10 ml of cold ADS buffer containing porcine pancreatic (1 mg/ml; Sigma) and collagenase type 2 (0.5 mg/ml; Worthington, Lakewood, NJ) in a sterile 250-ml Erlenmeyer flask and rotated in an incubator/shaker (100 rpm, 37°C, 30 min; Innova 4080 Shaking incubator; New Brunswick Scientific, Edison, NJ). The supernatant was transferred to a 50-ml conical tube containing 2 ml of fetal bovine serum (AA4409W; Gemini Bio Products, Woodland, CA) to inactivate the enzymes, followed by resuspension in 25 ml of ADS buffer. Ten milliliters of fresh ADS buffer containing the digestive enzymes were added to the cell pellet of the original flask for a second 30-min incubation while the first digest was centrifuged (1,200 rpm, 4°C, 15 min; Beckman TJ-6), and the cell pellet was resuspended in 2 ml of ADS buffer. The second digest was processed as before and combined with the resuspended pellet of the first digest. One milliliter of the cell suspension was layered over a Percoll gradient in a 15-ml conical tube (upper density, 1.058; bottom density, 1.082) and subjected to centrifugation (3,000 rpm, 4°C, 30 min). The top band of cardiac fibroblasts and lower band of cardiomyocytes were separately removed using sterile Pasteur pipettes, and the cells were stored on ice. ADS buffer was added to the Percoll gradient cells, and an additional centrifugation yielded a cell pellet (1,200 rpm, 4°C, 15 min; Beckman TJ-6). This pellet was resuspended in 8 ml of ADS buffer and subjected to a second Percoll gradient centrifugation yielding discrete fibroblast and cardiomyocyte bands that were collected. Cells from each gradient centrifugation were counted by hemocytometer and plated in equivalent numbers on culture plates coated with denatured collagen (0.7% gelatin; Difco, Kansas City, MO) at 37°C.

Cardiac myocyte cell culture model. Cardiomyocytes were seeded at clonal (25–100 cells/60-mm plate) or subconfluent densities (5–10 × 10^4 cells/100-mm plate) in parallel plates so that immunocytochemical analysis and precise population counts or doubling times could be calculated to determine the growth rates and purity of the preparation throughout the study. All cardiac cells were grown on collagen in medium comprising 10% horse serum (AMG16770; Invitrogen) and 5% fetal bovine serum in Dulbecco’s modified Eagle’s medium (DMEM)-medium 199 (4:1) at 37°C (10 ml/plate). A complete medium exchange was performed every 24 h to minimize conditioning effects by the cardiomyocytes. Cells from the fibroblast band were plated at high densities (2 × 10^5 cells/100-mm plate) to achieve confluence, at which time the medium was left unchanged for a minimum period of 3 days. At that time, the conditioned medium was removed and filtered to remove cells and particulate matter (0.22-μm syringe filter). Conditioned medium was used immediately after collection and filtration or frozen for subsequent use. All media were allowed to undergo only one freeze-thaw cycle for inclusion in this study. Bead-based multianalyte proteomics analysis and enzyme-linked immunosorbent assays (ELISA) were performed on media utilized in this study before their use in cell cultures, including determination of the acid-activated form of TGF-β1 (R&D Systems, Minneapolis, MN).

Experimental paradigm. Preliminary studies indicated that plating cells from the second digest after Percoll gradient centrifugation provided a cardiomyocyte purity ranging from 95 to 99.1% of the total cells present after 24 h. Thus all experiments were performed only on cardiomyocytes from this subset of the preparatory protocol and meeting this criterion. Cell density was determined every 24 h for 144 h by counting a minimum of 20 random fields per plate and extrapolating the average population to the area of the entire plate. Parallel serial plates were fixed each day and double stained for myosin heavy chain (MyHC) and vimentin to confirm cardiomyocyte and fibroblast composition, respectively, based on cell counts and immunocytochemical phenotype. Cardiomyocytes were often binucleate, and the number of cells falling into this classification was also monitored. The fibroblast cell band obtained from the Percoll gradient was examined after serial clonal and mass population platings and selected to contain predominantly fibroblasts (>99%). In rare cases, endothelial cells were identifiable by formation of a cobblestone colony phenotype, and differential preplating was repeated to purify the fibroblast population.

To analyze potential paracrine effects of fibroblasts on cardiomyocyte behavior, we mixed fibroblast-conditioned medium (≥72 h of conditioning) or PBS 1:1 with standard medium containing twofold concentrations of horse and fetal bovine serum to maintain a standard serum concentration. This medium was used to fed cardiomyocytes 24 h after plating and was changed daily for 144 h. The assignment of cells to receive standard, conditioned, or PBS control medium was performed randomly on plates seeded with equivalent amounts of cardiomyocytes derived from the second digest. The experimental paradigm meeting the criterion for cardiomyocyte culture purification (≥95%) was successfully repeated on five independent ventricular harvests performed at different times as well as an expanded experiment in which ventricles derived from six litters of neonatal rats were concomitantly harvested, pooled, purified, and subjected to the identical experimental protocol comparing the effects of standard and conditioned media.

Electron microscopy-ultrastructural analysis. Cultured cells from each experimental condition were detached with trypsin-EDTA (GIBCO Invitrogen) and collected by serial rinse in 1× PBS as previously described (22). The suspended cells were centrifuged in a 1.5-ml tube (1,200 rpm; Beckman TJ-6) to obtain a pellet, and the supernatant was replaced with 3% glutaraldehyde for overnight fixation at 4°C. Postfixation was performed in 1% osmium tetroxide for 90 min, followed by dehydration with ethanol (50%, 30 min; 70%, 30 min; 95%, 2 × 30 min; 100%, 2 × 30 min) and acetone (2 × 30 min). The pellet was then slowly infiltrated with epoxy resins (EM-Resin, Leica, Vienna, Austria) and embedded, and heated overnight at 65°C. Thin sections (~90 nm) were cut, stained with uranium and lead salts, and examined in a Philips CM 10 electron microscope.

Immunocytochemical specification of cell phenotype. All plates were rinsed twice in cold PBS and fixed in AFA for immunocytochemical analysis as previously described (22). Expression of MyHC was determined by immunelabeling with MF20, a pan-MyHC monoclonal antibody mixed 1:1 with BA-G5 (provided by Dr. S. Schiaffino, Universita degli Studi di Padua, Padua, Italy), an antibody specific for α-cardiac MyHC (1, 38). An indirect immunostaining procedure was used, incorporating an alkaline phosphatase-conjugated secondary antibody (1:500 dilution, IgG, goat anti-mouse; Sigma) followed by Western blue stabilized substrate (Promega, Madison, WI) as a chromogen in levamisole (125 mM) to block endogenous phosphatase activity. Similarly, a monoclonal antibody specific to connexin 43 associated with gap junction formation was employed (1:100; Sigma),
utilizing a horseradish peroxidase-conjugated secondary antibody (1:500 dilution, goat anti-mouse; Sigma) and visualized using diaminobenzidine tetrahydrochloride staining (22, 38). The presence of vimentin was determined using monoclonal antibody (clone V9) (1:250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) with a biotinylated secondary antibody (1:500 dilution, goat anti-mouse, Sigma). A streptavidin-alkaline phosphatase tertiary reagent (1:500 dilution; Zymed Labs, San Francisco, CA) was subsequently employed and visualized through the use of Fast red substrate in naphthol phosphate buffer (Sigma). An α-smooth muscle actin specific monoclonal antibody, clone 1A4 (1:100 dilution; Sigma) conjugated to alkaline phosphatase was utilized, incorporating an indirect immunostaining procedure with Vulcan red (Biocare Medical, San Diego, CA) serving as the chromogen. Initial platings of purified but untreated fibroblasts (V9, MF20/BAG5−) and cardiomyocytes (V9−, MF20/ BAG5+) were analyzed in parallel with antibodies specific to cardiomyocytes (MF-20/BAG5; MyHC) or fibroblasts (V9; vimentin) as exclusionary controls for the immunocytochemical assay as well as a check for cell purity (4, 22).

Images of individual cardiomyocytes positive for MyHC expression were acquired randomly from 60-mm plates seeded at clonal density containing 1) untreated controls in standard cardiac medium and 2) parallel cultures treated with fibroblast-conditioned medium. Each untreated plate was paired with a treated plate obtained from the same cell harvest and an identical lot of conditioned medium. Five different cell expansions and unique lots of conditioned medium were studied for 144 h. Two plates represented each condition and time point, including the “zero” time point, while nine plates represented each condition and time point in the pooled cardiomyocyte study. A minimum of 25 cardiomyocyte images was obtained from each plate and imported into the NIH Image analysis package for calculation of cell surface area (http://rsb.info.nih.gov/nih-image). The outer membrane border of each cardiomyocyte image was manually inscribed with a digital cursor, and the area demarcated by the tracing was calculated with a macrosubroutine (Image J) calibrated before use against a micrometer slide image captured at the same magnification as the cell image. Surface area was determined for cells from control and conditioned media at each time point.

Confocal imaging for determination of protein coexpression. Confocal images of cultured cardiomyocytes were obtained after immunofluorescent labeling with both myosin-and vimentin (rabbit polyclonal H84; Santa Cruz Biotechnology)-specific antibodies to ascertain coexpression of these proteins. A Leica DM RXI upright microscope attached to a TCS SP2 AOBS confocal system (Leica Microsystems, Nussloch, Germany) was employed for the analysis. Images were obtained directly from cells fixed with AFA in their respective culture plates by using a long working distance ×63, 0.90 numerical aperture, water-immersion objective. The secondary antibody for myosin was an IgG-fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma), whereas the vimentin secondary was a goat anti-rabbit phycoerythrin (PE)-conjugated antibody (Sigma). FITC (maximum excitation/maximum emission 495/519 nm)-labeled myosin and PE (maximum excitation/maximum emission 565/576 nm)-labeled vimentin were visualized using excitation wavelengths of 488 and 594 nm, respectively. The corresponding emission detector widths were set at 498–568 nm and 608–710 nm. Laser settings were adjusted to remove overlap between detector channels. In addition, the cells were imaged with standard transmitted light.

Multiplex bead and ELISA analysis of standard and conditioned media. Fluorokine MultiAnalyte Profiling (xMAP) using the Luminex 100 platform (Luminex, Austin, TX) was employed to measure levels of 24 defined small proteins in conditioned and unconditioned media samples. The technology incorporated polystyrene microspheres dyed internally with differing ratios of two spectrally distinct fluorophores to create a family of different spectrally addressed bead sets (catalog no. RCYTO-80K-PMX, Lincoplex kit; Linco Research, St. Charles, MO). Each bead set utilized in this study was conjugated with a biotinylated capture antibody specific for a unique rat cytokine/chemokine target, including eotaxin (eosinophil chemotactic protein: small inducible cytokine A11; CCL11), interleukins (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IL-18), IP-10 (small inducible cytokine B10: Cxcl10), TNF-α, interferon (IFN)-γ, GM-CSF (Csf2), GRO/KC (growth-regulated α-protein: Cxcl11), VEGF, G-CSF (granulocyte colony-stimulating factor), leptin, RANTES, MIP-1α, and MCP-1 (small inducible cytokine A2: CCL2). The assay was performed without antecedent serum depletion with a sensitivity ≤0.1 pg/ml. Analysis was performed on five samples of medium conditioned by fibroblasts derived from separate ventricular harvests. Results were compared with standard cardiac medium placed on gelatin-coated plates in the absence of cells and incubated under identical culture conditions for ≥72 h. The assays utilized a 96-well microplate format and were processed according to the manufacturer’s protocol, including generation of a standard curve for each target prepared in background medium diment (DMEM) over a fourfold range of dilution from 4.9 to 20,000 pg/ml, except for leptin, which was calibrated from 24.41 to 100,000 pg/ml. A value of 3.8 pg/ml was accepted as the lowest threshold for sensitivity, because this value fell within the linear range of the calibration assays. Standards and test media were pipetted at 25 μl per well in duplicate and mixed with 25 μl of the bead mixture. The microplate was incubated overnight at 4°C on a microtiter shaker. Wells were then washed with buffer (3 times) using a vacuum manifold. A secondary antibody cocktail was added to all wells and incubated for 2 h with agitation at room temperature. Streptavidin-PE was added to the wells and incubated for 30 min with constant shaking at room temperature. Wells were washed twice, assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system and Bio-Plex Manager software 4.0 (Bio-Rad Laboratories, Hercules, CA). Quantities were determined by comparison to standard curves obtained for each analyte.

An ELISA was performed to assay TGF-β levels via a quantitative sandwich immunoassay with the target monoclonal antibody precoated on a 96-well microplate (Invitrogen, Carlsbad, CA). Standards, controls, and experimental samples were added to wells according to the manufacturer’s protocol, and the data calculated were compared with a standard calibration curve that also defined the dynamic range of the assay. The presence of TGF-β in control and test samples was determined by its binding to the immobilized antibody. Unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for the cytokine of interest was added to the wells to sandwich the immobilized cytokine from the primary incubation step. Any unbound antibody enzyme reagent was then washed away. A chromogen solution was then added to the wells, and color developed in proportion to the amount of bound cytokine. The optical density was determined using an automated plate reader at 450 nm (Biotec, Winooski, VT) with a KC Junior program set to ±450 nm.

RESULTS

Changes in morphological and functional phenotype. The seeded cardiomyocyte cells exhibited shapes ranging from rodlike to round in the initial hours after plating, regardless of seeding density. Within 24 h of initial culture, the cardiomyocytes individually attached, flattened on the denatured collagen surface, and exhibited spontaneous rhythmic contractions. Isolated cells exhibited active membrane ruffling and extrusion of pseudopodial extensions, often forming fixed intercellular contacts with nearby cardiomyocytes. Individual cardiomyocytes and those within complex syncytial multiclesular structures were easily identified by their rhythmic contractions, with a substantial number exhibiting a typical cardiac binucleate phenotype. No statistical differences in proliferation of MyHC-

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positive cells were detected using two-way analysis of variance (ANOVA) to compare medium treatment and time in culture across the five independent experiments (n = 3 harvests: 10 × 10⁴ cells/plate; n = 2 harvests: 5 × 10⁴ cells/plate) or within the single expanded pooled analysis (7 × 10⁴ cells/plate) (Fig. 1, A and B). Serial analysis of individual cardiomyocytes plated at clonal density established that a small percentage of cells underwent division, yielding two cell clones within the first 2 h (12 of 187 cells) to 48 h (12 of 180 cells) after plating, but there were no significant differences in clonal frequency due to media conditions. No further clonal doublings were detected throughout the 144-h analysis period (i.e., no 4-cell clonal colonies were detected at any subsequent time point). Immunocytochemical analysis of MyHC and vimentin expression by double staining in mass culture plates fed with standard medium confirmed that MyHC and vimentin antibodies stained exclusionary populations and that >95% of the cells were MyHC positive upon initial plating (Fig. 2A).

There were evident differences in the contractile behavior and surface area of the cardiomyocytes with increased time in culture associated with differences in the media. Cardiomyocytes maintained in cardiac fibroblast-conditioned medium became progressively quiescent, with only rare foci of beating cells apparent by 96 h. These cells contracted unpredictably at low frequencies compared with the actively contracting parallel cultures maintained in standard or PBS-diluted medium for up to 144 h. Video excerpts of control and fibroblast-conditioned cultures depicting the differences in contractile behavior after 96 and 144 h of culture have been deposited at http://bioinformatics.upmc.edu/laframboise/papers/fibromedia.htm. Based on two-way-ANOVA, both medium constitution (P < 0.0001) and culture duration (P < 0.0008) had a significant effect on cardiomyocyte surface area, which increased at 24, 72, and 144 h of culture time (Fig. 3). Post hoc testing via the Student’s paired t-test indicated that cell surface area was significantly larger in cardiomyocytes maintained in condi-

Fig. 1. A: proliferation curves for cardiomyocyte cultures. The results were obtained from 3 separate parallel expansions of cardiomyocytes seeded equivalently at 10 × 10⁴ cells/100-mm plate and randomly assigned for culture in either standard cardiac medium (■), medium obtained after conditioning on cardiac fibroblast plates (□), or cardiac medium diluted with PBS but with horse serum concentrations equivalent to standard medium (×). Duplicate plates were fixed and stained for myosin heavy chain (MyHC), and positive cells were counted every 24-h increment to obtain the curves depicted. The results were compiled as means ± SD of the cell counts. There were no statistically significant differences in cell numbers regardless of treatment at any of the time points. Similar results were obtained for 2 experiments when the initial cell titers were at a density of 5 × 10⁴ cells/100-mm plate. B: immuno-

Fig. 2. A: immunocytochemical analysis of cultures in standard medium. Monoclonal antibodies specific to vimentin or MyHC were employed to determine the number of cardiomyocytes and fibroblasts present during a typical 144-h expansion after initial seeding with either 50,000 or 100,000 cells. Values are means ± SD for n = 5. B: immunocytochemical analysis of cultures in conditioned medium. The number of cells containing vimentin increased markedly beginning around 72 h of culture and approached the same level of expression by the end of the experiment as was seen for myosin-positive cells. Each point represents the mean ± SD of cells positive for either vimentin or myosin at each time throughout the study. Comparisons across standard and conditioned media demonstrated a significant difference in the number of vimentin-positive cells from 72 (P < 0.00025) through 144 h (P < 0.015).
tioned medium compared with those in standard medium at the same time point in 11 of the 12 sets of matched samples ($P < 0.05$) utilized for this analysis, with the one exception occurring at 144 h of culture when size variability was most profound.

Ultrastructural analysis (144 h) demonstrated that disorganized myosin-containing sarcomere structures were present in both control and treated cardiomyocytes (Fig. 4). Mitochondria were plentiful under both conditions, but treated cells displayed aberrant sizes and shapes as well as mitochondrial degradation compared with controls. Large glycogen depots were common among treated cells, whereas glycogen was diffusely distributed among most control cells. Some cardiomyocytes in each of the culture conditions developed vacuoles in the perinuclear zone, but this trait was exacerbated in PBS. Nevertheless, these cells continued to display rhythmic contractions. Connexin 43 was detected among these cells as punctate deposits throughout the cytoplasm or as aggregates at intercellular membrane contacts. It has previously been established that α-smooth muscle actin expression emerges in a subset of adult and neonatal cardiomyocytes in culture (9, 14). Immunocytochemical analysis indicated that this phenomenon occurred in the present study as well (Fig. 1B). Within the context of the pooled study, the number of cardiomyocytes expressing smooth muscle actin was significantly higher at each time point in conditioned medium compared with controls, affecting 60% of the total population by 144 h. Cells positive for MyHC and connexin 43 grown in fibroblast-conditioned medium displayed a large diversity of cell diameters and shape characteristics by 72 h that persisted for the duration of the study (Figs. 5 and 6).

A unique finding was that cardiomyocytes in cultures fed fibroblast-conditioned medium demonstrated a marked increase in the number of vimentin-positive cells at 72 h (means ± SD: control, $5.3 ± 2.9\%$ vs. conditioned, $46.3 ± 6.0\%$, $P < 0.00025$), far exceeding that seen in the other cultures and not accounted for by the small resident population of fibroblasts present at initial plating (Fig. 2B). The population of cells expressing vimentin in this cohort remained elevated for the duration of the study compared with other culture conditions. Upregulation of vimentin expression was contemporaneous with cessation of contraction as well as apparent diminution of intracellular myosin expression as evidenced by reduced staining intensity (Fig. 2B and 5D). However, the total number of myosin- and connexin 43-positive cells in conditioned medium was not statistically different compared with other culture conditions despite the diminution in myosin staining of individual cells (Fig. 1B).

Immunocytochemical staining for myosin often exhibited refractive birefringence consistent with an organized myosin macromolecular structure despite significant structural changes including development of cellular holes, cavities, and vacuoles (Fig. 6A). This was seen in fibroblast-conditioned medium as well, where few cells displayed contractions. The shape of the cardiomyocyte depicted in Fig. 6B was typical of each culture; however, the presence of vimentin in binucleate cells like this

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**Fig. 3.** Surface area of cardiomyocytes based on culture conditions. Images of individual cardiomyocytes plated at clonal densities were captured at identical magnification, the cell membrane border was manually traced, and the surface area was calculated using a calibrated algorithm (http://rsb.info.nih.gov/nih-image) (see MATERIALS AND METHODS). All paired comparisons at 24 and 72 h between conditioned and unconditioned cells representing 4 different cardiomyocyte types were significantly different by paired t-test ($P < 0.05$), as were 3 of 4 comparisons at 144 h ($P < 0.05$). Statistical significance was obtained between the mean ± SD for each group at each time point using an unpaired Students t-test.

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**Fig. 4.** Ultrastructural changes associated with conditioned medium. Cardiomyocytes from standard and conditioned media were analyzed by electron microscopy for ultrastructural changes. A: a cardiomyocyte grown under standard conditions for 6 days. B: a cell grown under identical conditions except for addition of fibroblast-conditioned medium. Gly, glycogen; Mi, mitochondria; N, nucleus; My, myosin in myofibrillar aggregates. Both images were obtained at 6,610 × 1,000 magnification.
specimen was predominantly restricted to fibroblast-conditioned medium. Thus conditioned medium induced a unique vimentin expression pattern in cardiomyocytes atypical of the other culture conditions employed in this study. By serial immunocytochemical analysis, it was established that the actin-positive cells also expressed connexin 43 and retained their capacity to form gap junctions with other cardiomyocytes regardless of the media conditions (Fig. 6C).

Confocal image analysis confirmed that myosin and vimentin were coexpressed within individual cells grown in fibroblast-conditioned medium (Fig. 7C). Myosin was typically organized into filamentous structures among the cells that retained a sarcomeric organization (Fig. 7A). Vimentin displayed this birefringent pattern in those cells as well but was typically distributed in an amorphous pattern throughout the cytoplasm, except in the nucleus (7B). It is important to note that a few cardiomyocytes retained their original phenotype of exclusionary myosin expression throughout the 6-day exposure to the fibroblast-conditioned medium. At the same time, rare cells were detected under standard cardiomyocyte growth conditions in which myosin and vimentin were coexpressed. Nevertheless, a dominant phenotype emerged among those cardiomyocytes exposed to fibroblast-conditioned medium where both myosin and vimentin were routinely present. Thus medium conditioned by factors specific to cardiac fibroblasts appeared to markedly amplify and/or select for this phenotype compared with the other conditions employed in this study.

Cytokine analysis of cardiac fibroblast-conditioned medium. Multianalyte profiling of unconditioned medium vs. conditioned medium obtained from five different cardiac fibroblast harvests revealed several cytokines at significantly elevated levels after exposure to fibroblast cultures compared with standard medium (Table 1). Ten molecular factors (VEGF, GRO/KC, MCP-1, leptin, MIP-1α, IL-6, IL-10, IL-12p70, IL-17, and TNF-α) were at or below threshold levels of...
detection in the unconditioned medium but were significantly elevated in the fibroblast-conditioned medium. RANTES was detectable above threshold levels in the unconditioned medium but rose to significantly higher levels in the fibroblast-conditioned medium. Of the 25 cytokines assayed, only GM-CSF was decreased in medium after exposure to cardiac fibroblasts compared with the unconditioned medium. ELISA analysis indicated that the conditioned medium had comparable levels of active TGF-β1 compared with the unconditioned medium conditions antecedent to use in culture (Table 1). However, ELISA performed after acid activation (pH 4) revealed that a significantly higher level of latent TGF-β1 was present in the

![Fig. 7. Confocal analysis of cardiomyocytes treated 6 days in conditioned media. A: a representative cardiomyocyte stained for myosin with a FITC secondary antibody. B: vimentin distribution in the same cell with a phycoerythrin secondary fluorophore. C: overlay of A and B indicating colocalization of myosin and vimentin (yellow color) within the same cell. D: standard transmission image of the cell.](image)

Table 1. Protein profiles of standard cardiac and fibroblast-conditioned media

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<th>Protein</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
<th>P Value</th>
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Twenty-four cytokines/chemokines were assayed from conditioned medium derived from 5 separate harvests of cardiac fibroblasts (n = 5) using a multianalyte bead panel. ELISA was used to detect TGF-β both before and after acid activation of the latent form (n = 4). (See MATERIALS AND METHODS for nomenclature and statistical analysis). The conditioned medium represents standard cardiac medium exposed to cardiac fibroblasts for a minimum of 72 h before collection and filtering to remove cells and debris. Asterisks indicate values at or below the linear range of detection based on the calibration curve for each protein assay. All values are expressed in pg/ml. P values represent the level of statistical significance obtained in each comparison with a cutoff for significance at P < 0.05. NS indicates values that were not significant.
conditioned medium compared with the standard ($P < 0.01$) or PBS control medium ($P < 0.01$).

**DISCUSSION**

The addition of cardiac fibroblast-conditioned medium to purified cardiomyocyte cultures was associated with 1) cell hypertrophy, 2) vimentin expression in the presence of MyHC, and 3) markedly reduced spontaneous rhythmic contractions intrinsic to untreated cultured cardiomyocytes. These effects on cardiomyocyte structure and chronotropy in fibroblast-conditioned medium differed both in nature and time frame from the dedifferentiation or “fetal” gene program previously delineated in cardiomyocytes cultured under standard conditions (9, 11–14). For example, the progressive emergence of α-smooth muscle actin across all culture conditions was consistent with the dedifferentiation paradigm in contrast to vimentin expression specific to the fibroblast-conditioned medium (9, 14). These data suggest that cardiac fibroblasts introduced a soluble factor or factors into the medium that altered cardiomyocyte function and phenotype by either 1) a direct cell membrane or intracellular interaction or 2) sequestration, consumption, or modification of factors in the standard medium, leading to an indirect biological effect. Further studies are required to dissect the specific factors and pathways underlying the in vitro cardiomyocyte response and to determine whether the paradigm applies to the complex in vivo environment.

Recent studies have emphasized the complexity of cardiac intercellular interactions where heart cells may be both source and target of signals such as cytokines and growth factors (16, 17, 31, 36). Contact-specific and density-related effects have been previously described for cardiomyocytes cocultured with fibroblasts, but the contribution of each cell fraction was difficult to interpret, particularly in light of recent evidence that cardiomyocytes can fuse with fibroblasts in vitro (12, 13, 27, 42). Therefore, we optimized our purification methods and immunocytochemical assays to restrict and monitor the presence of fibroblasts vs. cardiomyocytes by eliminating those cultures and conditions where multiple cell types were present. Based on 1) the randomized treatment paradigm, 2) the immunocytochemical results obtained on parallel control plates, and 3) the daily serial cell counts including binucleate cells, effects of the conditioned medium were not explained by selective overgrowth of fibroblasts or amplification of other cell types. Furthermore, confocal analysis confirmed that typical exclusionary expression of myosin in cardiomyocytes and vimentin in fibroblasts under standard culture conditions was replaced by coexpression within a substantial number of cardiomyocytes treated with conditioned medium.

Vimentin expression has rarely been assayed in neonatal and adult cardiomyocytes in vitro and has been implicated as part of a dedifferentiation or developmental program largely because embryonic cardiomyocytes in vivo and in vitro express vimentin during a fetal transition period (12, 21, 28, 29, 31, 46, 52). The present study is the first to directly demonstrate the emergence of coexpression of vimentin and MyHC in a highly purified postnatal cardiomyocyte culture. The delayed in vitro onset of vimentin expression in myosin-containing cells under the influence of conditioned medium differed in pattern from the developmental myogenic program where vimentin expression preceded mature myosin expression. It is interesting to note that coexpression of myosin, smooth muscle actin, and vimentin is a defining characteristic of the myofibroblast cell type (8, 19, 39, 47–49). Myofibroblasts derive from fibroblasts, monocytes, or circulating progenitor cells under the influence of TGF-β and form granulation tissue, produce extracellular matrix molecules, and contribute to wound contraction (7, 19, 50). Although TGF-β was not elevated in the conditioned medium before use, latent TGF-β was significantly increased. Thus the latent form may have been proteolytically activated after cardiomyocyte binding inducing the canonical TGF-β signal pathway, or latent TGF-β may have directly activated NF-κB in a pathway associated with cellular transformation (2, 7, 19, 26, 50). Respecification of endothelial, epithelial, and muscle cell phenotype has been reported previously in vitro but has not been confirmed in vivo (18, 24, 35). Thus it is intriguing to consider that cardiomyocytes might be a source of myofibroblasts and participate in the scarification process if the plasticity demonstrated in the present study extends to the in vivo domain. Robust induction of myofibroblasts is known to occur after myocardial injury, so determination of whether these cells may derive from cardiomyocytes in a manner analogous to the present study will require precise in vivo lineage studies.

Cardiac fibroblasts are known to play a critical role in scar formation based on their prolific proliferative capacity as well as through production of extracellular matrix glycoproteins. The present study adds to a growing literature that cardiac fibroblasts also generate paracrine signals that may directly influence cardiomyocyte behavior. In an analogous approach, a cardiomyocyte cell-survival signal pathway activated in vitro by the secreted protein thymosin β4 has been identified to ameliorate the effects of myocardial infarct in mice (6). The present data also reinforce in vitro cardiomyocyte-fibroblast coculture studies delineating IL-6 as an activator of cardiomyocyte hypertrophy (16, 17). Although we were able to maintain equivalent pH, temperature, and serum conditions across all media conditions, we cannot rule out changes in ionic composition and osmolality associated with the conditioning protocol that could account for physiological changes in highly sensitive processes such as spontaneous contractility, nor can we rule out the possibility that fibroblasts consumed or altered critical molecules during medium conditioning, thereby engendering a cardiomyocyte response by their absence or modification. The proteomics analysis revealed minimal signs of substrate depletion, but this likely reflected selection of an assay targeting cytokines and chemokines secreted by fibroblasts rather than growth factors, ions, and metabolites consumed by these cells. Among the 26 proteins assayed, only GM-CSF was significantly decreased in the medium after exposure to fibroblasts. No direct effect of GM-CSF administration or diminution has been reported on cardiomyocyte phenotype or function to date, but GM-CSF is important in hematopoiesis, the immune response, and arteriogenesis, and knockout mice exhibit evidence of cardiac amyloidosis (40). Thus we recognize the importance of efforts directed at determining molecules that, by their elimination, could account for changes in cardiomyocyte phenotype.

It was interesting to note that the capacity for fibroblasts to produce the cytokine protein profile detected in the cardiac fibroblast-conditioned medium has previously been demonstrated at the gene expression level in synovial fibroblasts in both primary tissue and serial culture associated with inflam-
matory joint disease (36). Results from those studies provided evidence of a persistent proinflammatory or immunopathogenic response originating in or enhanced by fibroblasts that produced a cytokine and chemokine profile very similar to that of the present study. When the proteins in the present study were analyzed for correlation with canonical cell signal transduction networks (Ingenuity Pathway Analysis 4.0; Redwood City, CA), we were surprised to note that the proteomics profile fit equally well into four other important pathways with the same high statistical significance (P < 0.001) as the inflammatory/immune response pathway. These included signal networks associated with 1) cellular proliferation, 2) cell-to-cell activation, 3) cellular movement, and 4) apoptosis. The fact that these pathways are fundamental to in vivo cardiac remodeling lends support to the hypothesis that cardiac fibroblasts play an important effector role in modulating the repair process apart from activation of an acute phase response.

These data reinforce the use of well-defined, highly purified cardiomyocyte preparations in vitro for investigating intercellular paracrine interactions and cell plasticity. The effects of fibroblast conditioning—hypertrophy, loss of spontaneous beating, coexpression of MYHc and vimentin, and glycogen accumulation—could be separated from global differentiation changes attributable to the in vitro model based on the carefully controlled experimental paradigm. We recognize that a reduced culture preparation places greater interpretive dis-
tance from organismal biology, thereby requiring translational studies to determine whether the findings apply to adult cardio-
myocytes both in vitro and in vivo. Nevertheless, the data add to recent findings demonstrating both autocrine and paracrine effects of nonmyocardial cells on the structure and function of cardiomyocytes. Furthermore, they suggest novel influences of fibroblasts on cardiomyocyte phenotype and plasticity as well as on the formation of myofibroblasts.

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