Cardiac Fibroblasts Influence Cardiomyocyte Phenotype In Vitro

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ABSTRACT

Cardiac fibroblasts impact myocardial development and remodeling through intercellular contact with cardiomyocytes but less is known about non-contact, 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 media, 2) standard media diluted 1:1 with phosphate buffered saline (PBS) or 3) standard media diluted 1:1 with media conditioned ≥72 hours by cardiac fibroblasts. Serum concentrations were held constant under all media conditions and complete media exchange performed daily. Cardiomyocytes began contracting within 24 hours 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 hours in all conditions. Only cardiomyocytes in fibroblast-conditioned media stopped contracting by 72 hours. There was a significant, sustained increase in vimentin expression specific to these cultures (X± S.D.: 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, MCP-1, leptin, MIP-1α, IL-6, IL-10, IL-12p70, IL-17, TNF-α) at or below detection levels in unconditioned media that were significantly elevated in fibroblast-conditioned media. Latent-TGF-β and RANTES were present in unconditioned media but rose to higher levels in conditioned media. Only GM-CSF was present above threshold levels in standard media but decreased by fibroblast conditioning. These data indicated that, under the influence of fibroblast-conditioned media, cardiomyocytes exhibited marked hypertrophy, diminished contractile capacity and phenotype plasticity distinct from the dedifferentiation program present under standard culture conditions.
Key Words: cardiomyocyte, fibroblast, proteomics, myosin heavy chain, vimentin, myofibroblast, primary culture, dedifferentiation, phenotype, plasticity
INTRODUCTION

Ventricular cardiomyocytes are thought to undergo proliferation arrest soon after birth such that pathological conditions including hemodynamic overload and myocardial infarct typically result in cardiomyocyte degeneration, dedifferentiation or hypertrophy rather than myocardial regeneration (10-12,15,45,47). Consequently, the ventricular injury response is dominated by fibroblast proliferation and sustained scarring rather than restoration of functional myocardium (10,15,49). Recent discoveries 1) that postnatal cardiomyocytes exhibit limited proliferative capacity and 2) 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. IL-1β, IL-6, TGF-β as well as various undefined soluble factors, may be of non-myocardial, epicardial or myocardial in vivo origin and act through paracrine or autocrine pathways (16,17,32,37,41-43). 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...
non-contact cell culture paradigms to assess the role of soluble factors (12,13,17,42).

The present study examined the effect of media 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 or additional to the \textit{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 media but present at significantly higher levels in the conditioned media including vascular endothelial growth factor (VEGF), growth regulated alpha protein (GRO/KC), monocyte chemoattractant protein 1 (MCP-1), leptin, macrophage inflammatory protein 1 alpha (MIP-1\textsubscript{\alpha}), interleukins 6, 10, 12p70 and 17 (IL-6, IL-10, IL-12p70, IL-17) and tumor necrosis factor-alpha (TNF-\textsubscript{\alpha}). Furthermore, two factors present in standard media were significantly elevated after cardiac fibroblast conditioning including latent-transforming growth factor-\textbeta (TGF-\textbeta) and regulated on activation normal T cell expressed and secreted (RANTES). Only one factor, granulocyte colony stimulating factor (GM-CSF), fell significantly below concentrations obtained in standard media 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 using CO₂. The study was performed according to NIH Guidelines (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 IACUC approval (SVM03.00). The atria were removed and the ventricles placed in cold ADS buffer containing antibiotics (streptomycin & penicillin: 50 units /ml; Gibco BRL, Carlsbad CA, ampicillin: 100mg/ml; Sigma, St. Louis MO) and antimycotic amphotericin B: 5 ug/ml; Mediatech, Herndon, VA). The ventricles (n=20 to 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 pancreatin (1mg/ml; Sigma, St. Louis, MO) 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 Inc, Edison, NJ). The supernatant was transferred to a 50 ml conical tube containing 2 ml of fetal bovine serum (A44409W, 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 minute incubation while the first digest was centrifuged (Beckman TJ-6: 4°C, 15 min., 1200 rpm) and the cell pellet 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=1.082) and subjected to centrifugation (3000 rpm x 1462 x g, 30 min., 4°C). 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 (Beckman TJ-6: 4°C, 15 min., 1200 rpm). 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 to 100 cells/60 mm plate) or subconfluent densities (5 to 10 x 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 media comprising 10% horse serum (AMG16770, Hyclone, Logan, Utah) and 5% fetal calf serum (FCS) (A44409W, Gemini Bio Products, Woodland, CA) in Dulbecco’s Modified Eagle’s Medium (DMEM)/ Medium 199 (4:1) at 37°C (10 ml/plate). A complete media exchange was performed every 24 hours in order to minimize conditioning effects by the cardiomyocytes. Cells from the fibroblast band were plated at high densities (2 x 10^5/100 mm plate) in order to achieve confluence at which time the media was left unchanged for a minimum period of 3 days. At that time, the conditioned media was removed, filtered to remove cells and particulate matter (0.22 micron syringe filter). Conditioned media was used immediately after collection and filtration or frozen for subsequent use. All media was allowed to undergo only one freeze-thaw cycle for inclusion in this study. Bead based multi-analyte proteomics analysis and enzyme-linked immunosorbent assays were performed on media utilized in this study but prior to 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 hours. 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 hours for 144 hours 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 and vimentin to confirm cardiomyocyte and fibroblast composition respectively based on cell counts and immunochemical 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.

In order to analyze potential paracrine effects of fibroblasts on cardiomyocyte behavior, fibroblast conditioned media (≥72 hours of conditioning) or PBS was mixed 1:1 with standard media containing two-fold concentrations of horse and fetal bovine serum to maintain a standard serum concentration. This media was used to feed cardiomyocytes 24 hours after plating and was changed daily for 144 hours. The assignment of cells to receive standard, conditioned or PBS control media 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 5 independent ventricular harvests performed at different times as well as an expanded experiment in which ventricles derived from 6 litters of neonatal rats were concomitantly harvested, pooled, purified and subjected to the identical experimental protocol comparing the effects of standard versus conditioned media.

**Electron Microscopy-Ultrastructural Analysis:** Cultured cells from each experimental condition were detached with trypsin EDTA (Gibco BRL, Carlsbad, CA.) and collected by serial rinse in 1X PBS as previously described (22). The suspended cells were centrifuged in a 1.5 ml tube (1200 rpm, Beckman Model YJ-6) to obtain a pellet and the supernatant was replaced with 3% glutaraldehyde for overnight fixation at 4°C. Post-fixation was performed in 1% osmium tetroxide for 90 minutes, followed by dehydration with ethanol (50%:30 min, 70%:30 min, 95%:2 x 30 min, 100%:2 x 30 min) and acetonitrile (2 x 30 min). The pellet was then slowly infiltrated with epoxy resins (EMbed-812, Electron Microscopy Sciences, Hatfield, PA), 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 myosin heavy chain (MyHC) was determined by immunolabelling with MF20, a pan myosin heavy chain monoclonal antibody mixed 1:1 with BA-G5 (provided by Dr. S. Schiaffino, Universita degli Studi di Padova, Padova, Italy), an antibody specific for α-cardiac myosin heavy chain (1,38). An indirect immunostaining procedure was used incorporating an alkaline phosphatase conjugated secondary antibody (1:500, IgG, goat anti-mouse, Sigma, St. Louis, MO) 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, St. Louis, MO) utilizing a horseradish peroxidase conjugated secondary antibody (1:500, goat anti-mouse, Sigma, St. Louis, MO) visualized using diaminobenzidine tetrahydrochloride (DAB) staining (22,38). The presence of vimentin was determined using monoclonal antibody, V9, (1:250 dilution, Santa Cruz Biologics, Santa Cruz, CA) with a biotinylated secondary antibody (1:500 dilution, goat anti-mouse, Sigma, St. Louis, MO). A streptavidin-alkaline phosphatase tertiary reagent (1:500, Zymed Labs, San Francisco, CA) was subsequently employed and visualized through use of Fast Red substrate in naphthol phosphate buffer (Sigma, St. Louis, MO). An α-smooth muscle actin specific monoclonal antibody, clone 1A4, (1:100, Sigma, St. Louis, MO) conjugated to alkaline phosphatase was utilized incorporating an indirect immunostaining procedure with Vulcan Fast Red (Biocarta, 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: myosin heavy chain) 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 myosin heavy chain expression were acquired randomly from 60 mm plates seeded at clonal density containing 1) untreated controls in standard cardiac media and 2) parallel cultures treated with fibroblast-conditioned media. Each untreated plate was paired with a treated plate obtained from the same cell harvest and an identical lot of conditioned media. Five different cell expansions and unique lots of conditioned media were studied for 144 hours. Two plates represented each condition and time point including the “zero” time point while 9 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 prior to 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 Biologics) specific antibodies in order to ascertain coexpression of these proteins. A Leica DM RXE 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 using a long working distance 63X, 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 Chemicals, St. Louis, MO) while the vimentin secondary was a goat anti-rabbit phycoerythrin (PE) conjugated antibody (Sigma Chemicals). FITC (Exmax/Emmax 495/519 nm) labeled myosin and PE (Exmax/Emmax 565/576 nm) labeled vimentin were visualized using excitation wavelengths of 488 nm 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 Inc., 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 (cat# RCYTO-80K-PMX; Lincoplex kit, Linco Research, Inc. 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 1α, 1β, 2, 4, 5, 6, 9, 10, 12p70, 13, 17 and 18 (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 (tissue necrosis factor-α), IFN-γ (interferon gamma), GM-CSF(granulocyte-macrophage colony stimulating factor: Csf2), GRO/KC (growth regulated alpha protein: Cxcl1), VEGF (vascular endothelial growth factor), G-CSF (granulocyte colony stimulating factor), leptin, RANTES (regulated on activation normal T cell expressed and secreted), MIP-1α (macrophage inflammatory protein 1 alpha) and MCP-1 monocyte chemoattractant protein 1: small inducible cytokine A2: CCL2). The assay was performed without antecedent serum depletion with a sensitivity ≤ 0.1 picogram/ml. Analysis was performed on five samples of media conditioned by fibroblasts derived from separate ventricular harvests. Results were compared with standard cardiac media placed on gelatin-coated plates in the absence of cells and incubated under identical culture conditions for ≥72 hours. 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 media diluent (DMEM) over a four-fold 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 as 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 (3X) using a vacuum manifold. A secondary antibody cocktail was added to all wells and incubated for two hours with agitation at room temperature. Streptavidin-phycoerythrin was added to the wells and incubated for 30 minutes 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 assay was performed to assay TGF-β levels via a quantitative sandwich immunotechnique 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 in comparison to 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 (Biotech, 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 hours 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 syncitial multicellular 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 positive cells were detected by two-way analysis of variance (2-way-ANOVA) comparing media treatment and time in culture across the 5 independent experiments (n=3 harvests: 10 x 10^4 cells/plate, n=2 harvests: 5 x 10^4 cells/plate) or within the single expanded pooled analysis (7 x 10^4 cells/plate) (Figure 1A and 1B). Serial analysis of individual cardiomyocytes plated at clonal density established that a small percentage of cells underwent division yielding 2 cell clones within the first 24 (12 of 187 cells) to 48 hours (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 hour analysis period i.e. no 4-cell clonal colonies were detected at any subsequent time point. Immunocytochemical analysis of myosin heavy chain and vimentin expression by double staining in mass culture plates fed with standard media confirmed that MyHC and vimentin antibodies stained exclusionary populations and that >95% of the cells were MyHC positive upon initial plating (Figure 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 media became progressively quiescent with only rare foci of beating cells apparent by 96 hours. These cells contracted unpredictably at low frequencies compared to the actively contracting parallel cultures maintained in standard or PBS diluted media for up to 144 hours. Video excerpts of control and fibroblast-conditioned cultures depicting the differences in contractile behavior after 96 and 144 hours of culture are deposited at:

http://bioinformatics.upmc.edu/laframboise/papers/fibromedia.htm. Based on two-way analysis of variance (2-way-ANOVA), both media constitution (p<0.0001) and duration of culture (p<0.0008) had a significant effect on cardiomyocyte surface area which increased at 24, 72 and 144 hours of culture time (Figure 3). Post-hoc testing via the students paired t-test indicated that cell surface area was significantly larger in cardiomyocytes maintained in conditioned media compared to those in standard media treatment 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 hours of culture when size variability was most profound.

Ultrastructural analysis (144 hours) demonstrated that disorganized myosin containing sarcomere structures were present in both control and treated cardiomyocytes (Figure 4). Mitochondria were plentiful under both conditions but treated cells displayed aberrant sizes and shapes as well as mitochondrial degradation compared to controls. Large glycogen depots were common among treated cells while glycogen was diffusely distributed amongst 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 contraction in cultures fed with standard media or PBS control media.
Conditioned Media Effects on Immunocytochemical Phenotype. Immunocytochemical analysis indicated that all cultures demonstrated myosin heavy chain expression throughout the dominant population of cells comprising a binucleate phenotype and/or 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 demonstrated that this phenomenon occurred in the present study as well (Figure 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 media compared to controls affecting 60% of the total population by 144 hours. Cells positive for MyHC and connexin 43 grown in fibroblast-conditioned media displayed a large diversity of cell diameters and shape characteristics by 72 hours which persisted for the duration of the study (Figures 5,6).

A unique finding was that cardiomyocytes in cultures fed fibroblast-conditioned media, demonstrated a marked increase in the number of vimentin positive cells at 72 hours (X± S.D.: 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 (Figure 2B). The population of cells expressing vimentin in this cohort remained elevated for the duration of the study compared to other culture conditions. Up-regulation of vimentin expression was contemporaneous with cessation of contraction as well as apparent diminution of intracellular myosin expression as evidenced by reduced staining intensity (Figure 2B, 5D). However, the total number of myosin and connexin 43 positive cells in conditioned media was not statistically different in comparison to other culture conditions.
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 (Figure 6A). This was seen in fibroblast-conditioned media as well where few cells displayed contractions. The shape of the cardiomyocyte depicted in figure 6B was typical of each culture, however, the presence of vimentin in binucleate cells like this specimen was predominantly restricted to fibroblast-conditioned media. Thus, conditioned media 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 (Figure 6C).

Confocal image analysis confirmed that myosin and vimentin were coexpressed within individual cells grown in fibroblast-conditioned media (Figure 7C). Myosin was typically organized into filamentous structures among the cells that retained a sarcomeric organization (Figure 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 media. 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 media where both myosin and vimentin were routinely present. Thus, media conditioned by factors specific to cardiac
fibroblasts appeared to markedly amplify and/or select for this phenotype compared to the other conditions employed in this study.

**Cytokine Analysis of Cardiac Fibroblast-Conditioned Media.** Multi-analyte profiling of unconditioned versus conditioned media obtained from 5 different cardiac fibroblast harvests revealed several cytokines at significantly elevated levels after exposure to fibroblast cultures compared to standard media (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 media but were significantly elevated in the fibroblast-conditioned media. RANTES was detectable above threshold levels in the unconditioned media but rose to significantly higher levels in the fibroblast-conditioned media. Of the 25 cytokines assayed, only GM-CSF was decreased in media after exposure to cardiac fibroblasts compared to the unconditioned media. ELISA analysis indicated that the conditioned media had comparable levels of active TGF-β1 compared to the unconditioned media 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 conditioned media compared to the standard (p<0.01) or PBS control media (p<0.01).
DISCUSSION

The addition of cardiac fibroblast-conditioned media to purified cardiomyocyte cultures was associated with 1) cell hypertrophy, 2) vimentin expression in the presence of myosin heavy chain and 3) markedly reduced spontaneous rhythmic contractions intrinsic to untreated cultured cardiomyocytes. These effects on cardiomyocyte structure and chronotropy in fibroblast-conditioned media differed both in nature and timeframe 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 media (9,14). These data suggest that cardiac fibroblasts introduced a soluble factor or factors into the media 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 media leading to an indirect biological effect. Further studies are required to dissect the specific factors and pathways underlying the in vitro cardiomyocyte response and 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 versus
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 media 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 media.

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 myocytes 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 myosin heavy chain in a highly purified postnatal cardiomyocyte culture. The delayed in vitro onset of vimentin expression in myosin containing cells under the influence of conditioned media 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 media prior to 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 NFκB 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 if 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). While 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 that fibroblasts consumed or altered critical molecules during media conditioning thereby engendering a cardiomyocyte response by their absence or modification. The proteomics analysis revealed minimal sign 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 media 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, 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 media has previously been demonstrated at the gene expression level in synovial fibroblasts both in primary tissue and serial culture associated with inflammatory joint disease (36). Results from those studies provided evidence of a persistant proinflammatory or immunopathogenic response originating in or enhanced by fibroblasts which produced a cytokine and chemokine profile very similar to 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 4 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 myosin heavy chain and vimentin and glycogen accumulation—could be separated from global dedifferentiation changes attributable to the in vitro model based on the carefully controlled experimental paradigm. We recognize that a reduced culture preparation places greater interpretive distance from organismal biology thereby requiring translational studies to determine whether the findings apply to adult cardiomyocytes 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.
<table>
<thead>
<tr>
<th>PROTEINS</th>
<th>UNCONDITIONED MEDIA (pg/ml)</th>
<th>CONDITIONED MEDIA (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S. D.</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.8*</td>
<td>1.8</td>
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</tr>
<tr>
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**Table 1: Protein Profiles of Standard Cardiac and Fibroblast-Conditioned Media.** Twenty-four cytokines/chemokines were assayed from conditioned media derived from five separate harvests of cardiac fibroblasts (n=5) using a multi-analyte bead panel (see Methods). ELISA was used to detect TGF-β both prior to and after acid activation of the latent form (n=4). (See methods for nomenclature and statistical analysis). The conditioned media represents
standard cardiac media exposed to cardiac fibroblasts for a minimum of 72 hours prior to collection and filtering to remove cells and debris. The asterisk (*) indicates values at or below the linear range of detection based on the calibration curve for each protein assay. All values are expressed in picograms per milliliter (pg/ml). The 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.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1A: Proliferation Curves for Cardiomyocyte Cultures. The results were obtained from 3 separate parallel expansions of cardiomyocytes seeded equivalently at $10 \times 10^4$ cells /100mm plate and randomly assigned for culture in either standard cardiac media (Standard=■) media obtained after conditioning on cardiac fibroblast plates (Conditioned=□), or cardiac media diluted with PBS but with horse serum concentrations equivalent to standard medium (PBS=×). Duplicate plates were fixed, stained for myosin heavy chain and positive cells counted every 24-hour increment to obtain the curves depicted in the figure. The results were compiled as mean ± standard deviation 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 two experiments when initial seedings were at a density of $5 \times 10^4$ cells/100 mm plate.

Figure 1B: Immunocytochemical Analysis of Pooled Cardiomyocytes. Cardiomyocytes were pooled, purified and seeded at $7 \times 10^4$ across 18-100mm plates per time point for comparison of control versus conditioned media regarding myosin heavy chain (MyHC), connexin 43, α-smooth muscle actin expression. Values are mean ± standard deviation. Solid connector lines and markers indicate standard media and dashed lines with open markers are results obtained in conditioned media (MyHC: ■ and □, Connexin 43: ● and ○, Alpha smooth muscle actin:▲ and △). There were no significant differences in MyHC and connexin expression (2-way ANOVA) based on type of media or time in culture while expression of α-smooth muscle actin was significantly different regarding both variables ($p<0.001$).
**Figure 2A: Immunocytochemical Analysis of Cultures in Standard Media.** Monoclonal antibodies specific to vimentin or myosin heavy chain were employed to determine the number of cardiomyocytes and fibroblasts present during a typical 144 hour expansion after initial seeding with either 50,000 or 100,000 cells. Values are mean ± standard deviation for n=5.

**Figure 2B: Immunocytochemical Analysis of Cultures in Conditioned Media.** The number of cells containing vimentin increased markedly beginning around 72 hours 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 ± standard deviation 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 hours (p<0.00025) through 144 hours (p<0.015).

**Figure 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 calculated using a calibrated algorithm (http://rsb.info.nih.gov/nih-image) (see Methods). All paired comparisons at 24 and 72 hours between conditioned and unconditioned cells representing 4 different cardiomyocyte and fibroblast expansions were significantly different by paired T-test (p<0.05) as were 3 of 4 comparisons at 144 hours (p<0.05). Stars indicate statistical significance (p<0.05) obtained between the mean and standard deviation for each group at each time point using an unpaired Students T-test.
**Figure 4: Ultrastructural Changes Associated With Conditioned Media.** Cardiomyocytes from standard and conditioned media were analyzed by electron microscopy for ultrastructural changes. Panel A depicts a cardiomyocyte grown under standard conditions for 6 days while Panel B contains a cell grown under identical conditions except for addition of fibroblast-conditioned media. Gly: glycogen, Mi: mitochondria, N: nucleus, My: myosin in myofibrillar aggregations. Both images were obtained at 6.61 x 1000 magnification.

**Figure 5: Cell Immunotypes Under Culture Conditions.** The 6-day cultures were analyzed for myosin heavy chain and vimentin expression by simultaneous incubation with monoclonal antibodies or analysis in parallel plates. Panel A shows standard culture conditions containing Western Blue stained cardiomyocytes (CM) reactive with the MyHC antibody and a fibroblast (FB) stained with Fast Red as the chromogen for the vimentin antibody. Panel C represents a PBS media control and panel D demonstrates morphology of cardiomyocytes after 6 days in fibroblast-conditioned media. The scale bar displayed in panel C applies also to panels A and B. Please note that the scale bar for panel D indicates a magnification approximately 50% compared to the other panels. This adjustment was necessary to obtain a representative field for presentation after the effects of cell hypertrophy. Panel B is obtained from a control plate in which all processing was identical except for the exclusion of the primary antibodies.

**Figure 6: Cell Phenotypes in Fibroblast-Conditioned Medium.** Immunocytochemical results for cells 6 days after culture in fibroblast-conditioned medium. Panel A shows a cardiomyocyte with birefringent myosin bands evident through the use of a myosin heavy chain antibody (MyHC) antibody coupled to Western Blue substrate. Panel B demonstrates a binucleate cardiomyocyte positive for the vimentin specific monoclonal antibody (VIM) coupled to Fast Red chromogen. Panel C demonstrates connexin 43 (HRP-DAB staining) (CNXN-43)
expression at junctional membrane structures between fused myocytes and concomitant α-smooth muscle actin (SM-ACT) (Vulcan Red chromogen) within these cardiomyocytes.

**Figure 7: Confocal Analysis of Cardiomyocytes Treated 6 Days In Conditioned Media.**

Panel A shows a representative cardiomyocyte stained for myosin with a FITC secondary antibody. Panel B demonstrates vimentin distribution in the same cell with a phycoerythrin secondary fluorophore. Panel C represents the overlay of panels B and C indicating colocalization of myosin and vimentin (yellow color) within the same cell. The standard transmission image of the cell is depicted in panel D.
FIGURE 1A

PROLIFERATION ASSAY
MyHC Positive Cells

CELL NUMBER x 1000

n=3 Independent Expansions

0 24 48 72 96 120 144

HOURS IN CULTURE

FIGURE 1B

MyHC, CONNEXIN 43, SM ACTIN

0 24 48 72 96 120 144

HOURS IN CULTURE

Single Expansion x 6 Pooled Litters
FIGURE 3

EFFECT OF MEDIA ON CELL SIZE

Surface Area (μ2)

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Conditioned</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Hrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72 Hrs</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>144 Hrs</td>
<td></td>
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</tr>
</tbody>
</table>

* Denotes significant difference
FIGURE 4
FIGURE 5

A

CM

FB

B

CM

C

CM

D

CM

20μm

20μm
FIGURE 6