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Temporal alterations in cardiac fibroblast function following induction of pressure overload

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Abstract

Increases in cardiovascular load (pressure overload) are known to elicit ventricular remodeling including cardiomyocyte hypertrophy and interstitial fibrosis. While numerous studies have focused on the mechanisms of myocyte hypertrophy, comparatively little is known regarding the response of the interstitial fibroblasts to increased cardiovascular load. Fibroblasts are the most numerous cell type in the mammalian myocardium and have long been recognized as producing the majority of the myocardial extracellular matrix. It is only now becoming appreciated that other aspects of fibroblast behavior are important to overall cardiac function. The present studies were performed to examine the temporal alterations in fibroblast activity in response to increased cardiovascular load. Rat myocardial fibroblasts were isolated at specific time-points (3, 7, 14, and 28 days) after induction of pressure overload by abdominal aortic constriction. Bioassays were performed to measure specific parameters of fibroblast function including remodeling and

contraction of 3-dimensional collagen gels, migration, and proliferation. In addition, the expression of extracellular matrix receptors of the integrin family was examined. Myocardial hypertrophy and fibrosis were evident within 7 days after constriction of the abdominal aorta. Collagen gel contraction, migration, and proliferation were enhanced in fibroblasts from pressure-overloaded animals compared to fibroblasts from sham animals. Differences in fibroblast function and protein expression were evident within 7 days of aortic constriction, concurrent with the onset of hypertrophy and fibrosis of the intact myocardium. These data provide further support for the idea that rapid and dynamic changes in fibroblast phenotype accompany and contribute to the progression of cardiovascular disease.

Keywords

Fibroblast; Extracellular matrix; Migration; Proliferation; Fibrosis; Rat (Sprague Dawley)

Introduction

Increased cardiovascular load, such as seen during hypertension and aortic stenosis, results in extensive myocardial remodeling. Pressure overload promotes myocardial growth or hypertrophy that includes increased size of individual heart myocytes and expansion of the extracellular matrix (ECM). A large number of studies have provided important insight into the responses of cardiac myocytes to increased cardiovascular load and have begun to elucidate the mechanisms of load-induced myocyte hypertrophy (for recent reviews, see Heineke and Molkentin 2006; Machackova et al. 2006; Catalucci et al. 2008). Comparatively little is known about the regulation of fibroblasts by increased load and the contribution of the fibroblasts to load-induced myocardial dysfunction.

The fibroblasts are a major cellular component of the ventricular myocardium ranging between approximately 10 and 60% of the total cell population, depending on developmental stage and disease status of the animal (Banerjee et al. 2007). From a number of in vivo and in vitro studies, it is clear that increased load promotes a “pro-fibrotic” response in cardiac fibroblasts that includes increased production of ECM components and enhanced fibroblast proliferation (Chapman et al. 1990; Carver et al. 1991; Butt et al. 1995; Lee et al. 1999; MacKenna et al. 2000). Recent studies have suggested that alterations in other aspects of fibroblast function may also contribute to the progression of cardiovascular disease (Burgess et al. 2002; Marganski et al. 2003; Squires et al. 2005; Flack et al. 2006). Marked phenotypic alterations have been demonstrated in fibroblasts correlating to myocardial disease including changes in adhesion, migration and generation of tensile force. These studies also illustrated that phenotypic changes in fibroblasts are retained in isolated cells even after several passages in vitro (Burgess et al. 2002). To date, few studies have examined temporal changes that may occur in fibroblast phenotype accompanying load-induced myocardial remodeling. The present studies were carried out to evaluate fibroblast function at multiple time-points following induction of pressure overload in a rat abdominal aortic constriction model and to correlate potential changes to load-induced hypertrophy and fibrosis. Time-points for analyses were chosen prior to overt hypertrophy and fibrosis (3 days post-aortic constriction), at the onset of hypertrophy and fibrosis (7 days post-aortic

constriction) and during well-established hypertrophy and fibrosis (14 and 28 days post-aortic constriction). These studies illustrated that the ability of fibroblasts to interact with and remodel the extracellular matrix is rapidly enhanced following induction of increased cardiovascular load. Understanding the phenotypic alterations in cardiac fibroblasts will provide important insight into the cellular mechanisms of myocardial adaptation to increased load.

Materials and methods

Animal model

An aortic constriction model was used to induce pressure overload in adult rats (Siri 1988; Terracio et al. 1991). Eight-week-old adult male Sprague-Dawley rats (200-250 g body weight) were purchased from Harlan and housed in an AAALAC-approved animal facility. All animals were acclimated for 3–5 days prior to surgery and food and water were provided ad libitum. All experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and were approved by the University of South Carolina Institutional Animal Use and Care Committee (IACUC). For aortic constriction surgery, animals were anesthetized with a combination of ketamine hydrochloride, xylazine, and acepromazine administered intraperitoneally. A ventral abdominal laparotomy was performed to expose the abdominal aorta between the renal arteries. A 21-gauge needle was placed immediately adjacent to the aorta and a sterile 3.0 suture tied tightly around the needle and the aorta. The needle was carefully removed producing an aortic constriction. Abdominal muscles and skin were closed with a 3.0 absorbable suture and autoclips, respectively. Buprenorphine hydrochloride (0.025 mg/kg body weight) was given immediately following surgical procedures and again 6 h post-surgery if needed. Time-points for end assays were chosen prior to overt hypertrophy and fibrosis (3 days post-aortic constriction), at the onset of hypertrophy and fibrosis (7 days post-aortic constriction) and during well-established hypertrophy and fibrosis (14 and 28 days post-aortic constriction). After the appropriate periods, animals were euthanized by cervical dislocation following halothane inhalation. Sham control animals underwent similar surgical procedures without constriction of the abdominal aorta.

Assessment of cardiac hypertrophy

Heart weight to body weight and heart weight to tibia length ratios were determined as indicators of the progression of myocardial hypertrophy in the present studies. Following sacrifice, animals were weighed and hearts removed. Hearts were rinsed briefly and weights determined. Tibias were dissected and tibia length measured following removal of the soft tissue. Hearts were subsequently used for isolation of fibroblasts and for analysis of collagen accumulation.

Analysis of collagen accumulation

For evaluation of collagen accumulation in the myocardium, heart tissue was fixed in 2% paraformaldehyde and embedded in paraffin. Tissue sections were subsequently stained with picrosirius red (Histo-Scientific Research Laboratories, Woodstock, VA). Images of stained

slides were analyzed using ImagePro Plus (Media Cybernetics, Silver Spring, MD) and the collagen volume fraction determined.

Isolation of cardiac fibroblasts

Fibroblasts were isolated at 3, 7, 14, and 28 days following aortic constriction or sham surgery as described previously (Borg et al. 1983; Burgess et al. 2002). Following sacrifice of the animals, hearts were removed and rinsed in sterile saline. Extracardiac tissue was discarded and heart tissues were minced in sterile saline. Fibroblasts were isolated by enzymatic digestion of minced myocardial tissue with Liberase 3 (Roche Applied Science, Indianapolis, IN) in warm Krebs-Henslett Buffer (Burgess et al. 2002). Cells collected from digested tissue were plated into T75 flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 5% newborn calf serum, and antibiotics. Fibroblasts were purified by their rapid attachment to tissue culture plastic. Cells were passaged at approximately 80% confluency following detachment with a 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (trypsin/EDTA) solution. Fibroblasts were used prior to passage 3 and all experiments comparing cells were done with the same passage. Previous studies have illustrated that fibroblasts from pressure overload and other models retain phenotypic characteristics through at least 6 passages in vitro (Burgess et al. 2002).

Collagen gel contraction

The 3-dimensional collagen gel system has been extensively used to examine interactions between cardiac fibroblasts and the collagenous ECM (Burgess et al. 1994; Carver et al. 1995). Fibroblasts were detached from culture plates by incubation in trypsin/EDTA. Cells were rinsed, pelleted by centrifugation and resuspended in medium. An equal volume of cells was combined with a 1.25-mg/ml collagen solution to yield a final concentration of 100,000 cells/ml. Next, 1 ml of the collagen:cell mixture was added to wells of 24-well plates that had been precoated with bovine serum albumin. Collagen gels were allowed to polymerize 1 h at 37°C. Following polymerization, 1 ml of medium was gently added to each well of the plate and collagen gels dislodged from the well to allow the gels to float in the medium. Culture was continued for 48 h and collagen gels photographed. The relative contraction of the collagen gels was determined by measuring the top surface of the gels at 48 h relative to their initial size. Collagen gels were performed in triplicate with each of at least three independent cell isolations.

Fibroblast migration assay

The relative migratory ability of fibroblasts was determined using a scratch assay as previously described (Liang et al. 2007). Fibroblasts were plated onto wells of 6-well plates precoated with collagen type I (10 µg/ml). Cells were allowed to reach confluency before a scratch was gently created through the confluent cells with a pipet tip. The area devoid of cells was photographed at the time the scratch was created and at 12, 24, 36, and 48 h later. The relative migration of fibroblasts into the denuded area was measured using ImageJ (National Institutes of Health, Bethesda, MD). Migration experiments were performed in triplicate with each of at least three independent cell isolations.

Fibroblast proliferation

The proliferation of isolated cardiac fibroblasts was assayed by BrdU incorporation. For analysis of proliferation, cells were cultured on glass coverslips that were precoated with collagen type I. Culture was continued for 24 h with BrdU being added for the final 4 h of this period (Roche Applied Science, Indianapolis, IN). Following culture, coverslips were rinsed with phosphate-buffered saline and fixed in absolute ethanol containing 10 mM glycine (pH 2.0) for 30 min at -20°C . Cells were rinsed and BrdU incorporation detected by immunocytochemical staining with anti-BrdU serum and fluorescein-conjugated secondary antibodies. Cells were simultaneously stained with propidium iodide to detect all nuclei. Cells were examined and the percentage of BrdU-positive fibroblasts determined using a Nikon E600 microscope equipped for epifluorescence. Proliferation assays were performed on duplicate coverslips from each of at least three cell isolations.

Western blots

The effect of increased cardiovascular load on the expression of receptors for the ECM in isolated cardiac fibroblasts was determined by Western blot analysis. After 24 h of culture, fibroblasts were rinsed with sterile saline and total protein was extracted in lysis buffer containing 100 mM TRIS-HCl (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 0.5% NP 40, and protease inhibitors (Diaz-Araya et al. 2003). Following extraction, total protein content of the samples was determined using the BCA Assay (Pierce, Rockford, IL). Equal amounts of protein were separated on 5–15% gradient polyacrylamide gels (Pierce). Proteins were transferred onto nitrocellulose membranes and then incubated in primary antisera against $\beta 1$, $\alpha 1$, and $\alpha 2$ integrins (Chemicon, Billerica, MA). For normalization purposes, blots were also probed with antibodies against glyceraldehyde 3-phosphate dehydrogenase (Chemicon). Western blots were developed with SuperSignal reagent (Pierce) and exposed to X-ray film. Relative protein expression was determined by image analysis of X-ray films using an Alpha Innotech gel analysis system.

Analysis of mRNA expression

The relative expression of specific pro-fibrotic growth factors and cytokines was examined in isolated fibroblasts by semi-quantitative polymerase chain reaction. Fibroblasts were isolated and cultured as described above. Fibroblasts were rinsed with sterile saline and total RNA isolated using TriZol Reagent (Invitrogen, Carlsbad, CA). RNA was spectrophotometrically quantified and cDNA produced using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Polymerase chain reaction was carried out with primers specific for rat transforming growth factor- β (TGF- β), angiotensinogen, interleukin 6 (IL-6) or connective tissue growth factor and Taq polymerase (New England BioLabs, Ipswich, MA). Preliminary experiments were carried out to optimize conditions for PCR including cycle number for accurate quantification of products. Semiquantitative PCR was performed with RNA from fibroblasts isolated from six different sham or pressure overloaded rat hearts. Quantification of PCR products of experimental genes was normalized to that of acidic ribosomal phosphoprotein (PO), whose expression appears to be relatively consistent under different experimental conditions.

Statistical analysis

Results are shown $\pm$ SEM. Statistical significance was determined by comparison of unpaired *t* tests or ANOVA with Dunnett's post-hoc test as appropriate ($P < 0.05$). Analyses were performed using GraphPad Prism Software.

Results

Assessment of myocardial hypertrophy and fibrosis

Total heart mass relative to animal body weight and relative to tibia length were used as indicators of myocardial hypertrophy in the present studies (Table 1). These analyses illustrated a significant increase in the ratio of heart weight to the animal body weight (mg/g) and in the ratio of the heart weight to tibia length (mg/cm) by 7 days after abdominal aortic constriction (relative to the corresponding sham animals). These ratios remained elevated in the pressure-overloaded animals at 14 and 28 days post-surgery relative to corresponding shams.

Increased cardiovascular load also results in accumulation of ECM components, synthesized predominantly by the cardiac fibroblasts. Analysis of picrosirius red-stained heart sections was performed to determine the temporal progression of fibrosis in the pressure overloaded rat heart (Fig. 1). Increased staining with picrosirius red was seen in the myocardium after pressure overload (Fig. 1b and c) compared to the myocardium of corresponding sham animals (Fig. 1a). Quantitative image analyses indicated a significant accumulation of interstitial collagen by 7 days after aortic constriction in parallel to myocardial hypertrophy. The collagen volume fraction increased further at 14 and 28 days post-aortic constriction surgery (Fig. 1d).

Collagen gel contraction

Fibroblasts cultured in 3-dimensional collagen gels will, over time, remodel and contract the collagen matrix (Fig. 2a and b). The ability of fibroblasts isolated from sham and pressure overloaded hearts (3, 7, 14, and 28 days after aortic constriction surgery) to contract collagen gels was compared. These studies illustrated that, in general, fibroblasts isolated from hearts after aortic constriction surgery contracted collagen gels more effectively than cells from corresponding sham hearts (Fig. 2c, shorter bars indicate enhanced contraction). However, the increased collagen gel contraction was statistically significant only at 7 and 14 day time-points.

Fibroblast migration

A scratch assay (Fig. 3) was used to determine differences in migratory ability of cardiac fibroblasts from sham and pressure overloaded hearts. For these assays, a scratch was made through confluent cultures of fibroblasts and migration into the denuded area analyzed over time (Fig. 3a and b). In general, fibroblasts from the pressure overloaded rat hearts migrated into the scratched region of the culture plate more rapidly than the fibroblasts from the sham hearts. To quantify this, the relative migration of cells into the scratch over a 12-h period was measured in all of the cell types. Fibroblasts isolated from hearts at all time-points

following aortic constriction surgery had increased migratory activity compared to fibroblasts from corresponding sham hearts (Fig. 3c).

Fibroblast proliferation

Studies have indicated increased proliferation of interstitial fibroblasts in vivo following induction of pressure overload (Kuwahara et al. 2002; Sedmera et al. 2003). Incorporation of BrdU in vitro was used to assess proliferation of fibroblasts isolated at different times following pressure overload or sham surgery (Fig. 4a and b). In the present studies, proliferation of fibroblasts from pressure overloaded animals was significantly increased relative to fibroblasts from corresponding shams as early as 7 days after aortic constriction (Fig. 4a). This increase in proliferation was maintained at 14 days.

Integrin protein expression

Integrins represent the major family of cell surface receptors that mediate interactions of heart fibroblasts with the ECM (Terracio et al. 1991). In light of alterations described above in fibroblast migration and collagen contraction, Western blots of fibroblast protein lysates were performed to assay the expression of the $\alpha 1$ and $\alpha 2$ integrins, which are two of the major collagen receptors in cardiac fibroblasts. The relative levels of the $\alpha 1$ integrin protein showed a slight, but significant, increase in fibroblasts isolated from 3-day pressure-overloaded hearts relative to fibroblasts from corresponding sham animals (Fig. 5a). Interestingly, the expression of this integrin decreased significantly in the fibroblasts from 28-day pressure-overloaded hearts. The expression of the $\alpha 2$ integrin was significantly increased in fibroblasts from animals 7 and 14 days after aortic constriction (Fig. 5b).

Expression of profibrotic growth factors and cytokines

A number of biochemical factors play roles in the progression of myocardial hypertrophy and fibrosis in response to increased cardiovascular load. The cellular sources of all of these factors have not been identified; however, it has been suggested that fibroblasts play a sentinel role and secrete various biochemical factors in response to mechanical stimulation (Kaufman et al. 2001; Silzle et al. 2004). Several factors known to play a role in myocardial hypertrophy and fibrosis were assayed by semiquantitative PCR with RNA from fibroblasts isolated from 7-day sham and pressure-overloaded hearts (Fig. 6). These assays illustrated an increase in TGF- β and IL-6 in cardiac fibroblasts from pressure-overloaded hearts compared to those from sham hearts (Fig. 6). No significant difference was seen in the expression of angiotensinogen or connective tissue growth factor at this stage. This suggests that fibroblasts are an important source of these factors in the normal and overloaded heart and that the increased expression of these biochemical factors may, in part, be responsible for alterations in fibroblast behavior.

Discussion

While myocytes make up the bulk of the volume of the myocardium, fibroblasts are a major cell type in the adult heart and form an extensive network surrounding cardiomyocytes. The interstitial fibroblasts have been recognized for some time as the cell type responsible for producing and modifying the myocardial ECM. It is becoming increasingly clear that other

activities of fibroblasts can significantly impact cardiac development, function, and pathology (Baudino et al. 2006; Porter and Turner 2009). For instance, several recent studies have identified alterations in the adhesion between fibroblasts and the surrounding ECM concurrent with pathological myocardial remodeling. Fibroblasts from a pacing-induced left ventricular failure model demonstrated increased adhesion to ECM components including collagen type I, collagen type IV, laminin, and fibronectin (Flack et al. 2006). Likewise, fibroblasts isolated from a mouse myocardial infarction model were more adherent to select ECM components than cells from sham animals (Squires et al. 2005). These studies identified important functional differences in fibroblasts that correlated with pathological remodeling and illustrated that these differences are retained even after isolation and culture of fibroblasts. The present studies were undertaken to identify changes in fibroblast activity in response to increased cardiovascular load and to correlate these temporally to the progression of load-induced myocardial hypertrophy and fibrosis. These studies illustrated rapid and prolonged alterations in fibroblast proliferation, migration, and contractile activity that could significantly impact myocardial remodeling subsequent to increased cardiovascular load.

Increased cardiovascular load promotes an adaptive response that includes myocardial growth or hypertrophy and an expansion of the perivascular and interstitial ECM. The temporal analyses performed here illustrated significant myocardial hypertrophy by 7 days after partial constriction of the abdominal aorta. Analysis of heart sections stained with picrosirius red illustrated a significant increase in collagen accumulation at 7 days post-aortic constriction that progressed at 14 and 28 days. Neither hypertrophy nor fibrosis was apparent after 3 days of pressure overload. Alterations were evident in bioassays performed with fibroblasts from pressure-overloaded hearts compared to fibroblasts from corresponding sham hearts including increased proliferation, collagen gel contraction, migration, and expression of ECM receptors of the integrin family. Interestingly, some of these alterations in fibroblasts, particularly enhanced migratory activity, were evident at 3 days post-surgery, even prior to detectable myocardial hypertrophy or fibrosis. This suggests that relatively rapid alterations in fibroblast phenotype by increased load may play an important role in subsequent myocardial remodeling.

The interactions of cells with their surrounding matrix play critical roles in a number of cellular processes including migration, differentiation, gene expression, and apoptosis (Jane-Lise et al. 2000; Ingber 2006; Nelson and Bissell 2006). Several studies have suggested that interactions between fibroblasts and the ECM generate significant contractile forces that may impact diastolic function of the heart (Marganski et al. 2003; Sun and Weber 2000). The differentiation of fibroblasts into a more contractile myofibroblast phenotype has been correlated to pathological conditions including myocardial infarction and pressure overload hypertrophy (Leslie et al. 1991; Brown et al. 2005). In addition, fibroblasts isolated from spontaneously hypertensive rats are able to generate significantly more tensile force than cells isolated from control rats (Marganski et al. 2003). In the present studies, a 3-dimensional collagen gel model was used to determine whether fibroblasts isolated from sham and pressure-overloaded hearts differ in their ability to interact with the collagenous ECM. In this model, fibroblasts cultured within the collagen matrix will, over time, remodel and contract the collagen gels (Carver et al. 1995; Bell et al. 1979; Guidry and Grinnell

1987). The data presented herein illustrate that fibroblasts isolated from pressure-overloaded hearts contract collagen gels more effectively than fibroblasts from sham animals. This is in contrast to previous studies illustrating no differences in collagen gel contraction between fibroblasts isolated from sham and 10-week pressure-overloaded rat hearts (Burgess et al. 2002). This discrepancy may be due to the time-points selected for analysis of fibroblast function. Indeed, in the present studies, even as early as 3 days after induction of pressure overload, a trend towards enhanced collagen gel contraction was evident. These data suggest that fibroblast behavior is altered in a dynamic manner that may be reflective of changes in the mechanical properties and remodeling of the functioning heart (Ross and Borg 2001; Wang et al. 2003).

The major family of ECM receptors described to date in cardiac fibroblasts and many other cell types are the integrins (Terracio et al. 1991; Ross and Borg 2001). A number of studies have illustrated that integrins and integrin signaling are involved in myocardial hypertrophy; however, much of this work has focused on cardiomyocytes (Lu et al. 2006; Schluter and Wollert 2004). Integrins of the $\beta 1$ subfamily are critical to adhesion, migration- and collagen gel contraction by cardiac fibroblasts. Based upon these previous functional data, we anticipated that expression of specific integrins would be increased to correlate with enhanced collagen gel contraction- and migration in the present studies. The levels of the $\alpha 1$ integrin, a major receptor for collagen and laminin, were slightly increased in the fibroblasts from the pressure-overloaded animals only at 3 days post-surgery. The relative levels of $\alpha 1$ integrin were similar between sham and pressure-overloaded animals at 7 and 14 days after surgery and even decreased at 28 days post-surgery. The $\alpha 2$ integrin levels increased significantly in the fibroblasts from pressure-overloaded animals at 7 and 14 days post-surgery. The dynamic changes in collagen-binding integrins following pressure overload makes it tempting to speculate that the $\alpha 1$ and $\alpha 2$ integrins may mediate different aspects of myocardial remodeling; however, the significance of this remains to be determined. The increased expression of these collagen-binding integrins at 3, 7, and 14 days correlates to enhanced migration and collagen gel contraction by fibroblasts at these times. In contrast, neither of these integrins was increased in fibroblasts isolated from hearts at 28 days post-aortic constriction. Despite this, collagen gel contraction and migration were still enhanced. This suggests that other integrins or other ECM receptors may be mediating these processes in fibroblasts. Alternatively, differences in fibroblast proliferation within the collagen gels may affect contraction of the gels. While the number of fibroblasts initially seeded in the 3-dimensional collagen gels was the same in all experiments, it is possible that increased proliferation of cells from pressure-overloaded animals could impact collagen gel contraction. Further studies will be required to elucidate the mechanisms of enhanced collagen gel contraction by fibroblasts from pressure-overloaded hearts.

Numerous studies have demonstrated that the expression of ECM components and ECM-degrading enzymes by fibroblasts are altered in various cardiovascular diseases; however, only recently is it being appreciated that other functions of the fibroblast may impact ventricular remodeling. To our knowledge, the present studies are among the first to evaluate temporal changes in fibroblast function following induction of ventricular remodeling by increased cardiovascular load. These studies illustrate rapid and dynamic alterations in fibroblast activity including changes in contractility, migration- and

proliferation. It is becoming increasingly clear that changes in fibroblast function impact myocardial remodeling. The fact that these phenotypic changes are maintained in vitro provides novel opportunities to probe the molecular mechanisms of fibroblast function.

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Fig. 1.

Photomicrographs (a–c) and quantitative analysis (d) of picosirius red-stained myocardial sections from sham (S) and pressure-overloaded animals (PO). The micrographs illustrate comparable sections of the left ventricular wall in sham (a), 7-day pressure-overloaded (b) and 28-day pressure-overloaded (c) animals. Green-yellow indicates staining of the collagenous ECM. Quantitative analysis (d) of collagen staining indicates a significant increase in the collagen volume fraction in the left ventricular myocardium 7, 14, and 28 days post-aortic constriction ($*P < 0.05$ compared to corresponding sham). Scale bar equals 30 μ m

Fig. 2.

These show photographs of collagen gels containing fibroblasts from hearts of 14-day sham (a) and 14-day pressure-overloaded rat hearts (b). Note that the collagen gels are smaller with fibroblasts from the pressure overloaded animals (*dotted lines* outline the collagen gels). c Quantitative data. Note that fibroblasts from pressure-overloaded animals (PO) contract collagen gels more effectively than fibroblasts from corresponding sham (S) animals at all time-points (shorter *bars* indicate more contraction); however, this is significant only with fibroblasts from hearts 7 and 14 days after aortic constriction ($*P < 0.05$). Data from PO cells are presented relative to corresponding sham cells

Fig. 3.

These show photomicrographs illustrating the scratch migration assay with cardiac fibroblasts. *Dotted lines* illustrate the edges of the initial denuded area in the confluent culture (a) and the area remaining devoid of cells 24 h later (b). c Quantitative analysis of the migration of the cells into the denuded area. Fibroblasts from pressure-overloaded animals migrated more effectively in this assay ($*P<0.05$). Data are presented relative to the corresponding sham samples

Fig. 4.

These show examples of BrdU staining in fibroblasts isolated 7-day pressure-overloaded hearts (**a** and **b**). **a** Immunocytochemical detection of BrdU; **b** propidium iodide staining of all nuclei in the field. *Yellow arrows* indicate nuclei that are positive for BrdU (*green*) and propidium iodide (*red*), *white arrows* indicate BrdU-negative nuclei. The percentage of BrdU-positive fibroblasts from pressure-overloaded hearts was normalized to that of fibroblasts from sham hearts. **c** Enhanced proliferation of fibroblasts from pressure-overloaded animals relative to sham animals; however, this was statistically significant only at the 7- and 14-day time-points ($*P < 0.05$ compared to fibroblasts from sham animals). *Scale bar (b)* 25 μ m

Fig. 5.

Western blot analyses of $\alpha 1$ integrin (**a**) and $\alpha 2$ integrin (**b**) expression in fibroblasts isolated from 3-, 7-, 14-, and 28-day sham (S) and pressure-overloaded (PO) hearts. Quantification of specific integrins was normalized by reprobing Western blots with glyceraldehyde 3-phosphate dehydrogenase (as a loading control). Data are presented as the expression from fibroblasts in pressure-overloaded animals relative to expression in the corresponding sham fibroblasts. Expression of the $\alpha 2$ integrin was increased in fibroblasts from pressure-overloaded hearts 7 and 14 days post-surgery. In contrast to this, the expression of $\alpha 1$ integrin was slightly increased in fibroblasts from 3-day pressure-overloaded animals, but then decreased in the 28-day pressure-overloaded animals (* $P < 0.05$). *Insets* show representative blots with each antibody and lysates from sham and PO fibroblasts to illustrate the specificity of the antibodies

Fig. 6.

Semi-quantitative polymerase chain reaction (PCR) analysis of biochemical factors known to promote cardiac hypertrophy and fibrosis including transforming growth factor- β (*TGF- β*), *angiotensinogen*, interleukin-6 (*IL-6*), and connective tissue growth factor (*CTGF*). Semi-quantitative PCR was performed with RNA from fibroblasts isolated from 7-day sham or pressure-overloaded hearts (*PO*) and primers specific for the indicated factors. Relative levels of PCR product from the indicated factors were normalized to acidic ribosomal phosphoprotein. Data are presented as the expression in pressure overloaded fibroblasts relative to that of sham fibroblasts. Expression of *TGF- β* and *IL-6* were increased in fibroblasts from pressure overloaded hearts relative to that in fibroblasts from sham hearts (* $P < 0.05$)

Table 1

Body weights, heart weights and tibia lengths for sham and pressure-overloaded (PO) rats. Heart weight to body weight and heart weight to tibia length ratios were used as measures of myocardial hypertrophy. These are significantly increased in pressure-overloaded animals 7, 14, and 28 days post-aortic constriction

	Body wt (g)	Heart wt (mg)	Tibia length (cm)	Heart wt/body wt	Heart wt/tibia length
1 day sham	207.7±3.28	773.3±58.12	3.133±0.88	3.614±0.27	246.3±14.25
1 day PO	204.5±5.01	772.5±45.16	3.10±0.123	3.610±0.210	251.3±20.18
3 days sham	222.3±4.26	866.7±29.63	3.433±0.033	3.705±0.101	235.0±7.0
3 days PO	230.8±3.66	845.0±13.23	3.425±0.025	3.667±0.111	246.8±4.54
7 das sham	262.8±3.96	888.5±24.31	3.145±0.037	3.411±0.089	248.8±6.97
7 days PO	246.1±5.16	950.8±62.37*	3.147±0.047	3.895±0.119*	278.3±5.03*
14 days sham	272.8±4.96	862.5±40.70	3.375±0.048	3.159±0.112	255.3±8.67
14 days PO	266.7±10.3	1,090.8±72.3*	3.440±0.040	3.965±0.240*	316.4±19.20*
28 days sham	323.3±3.54	1,056.6±29.77	3.940±0.025	3.254±0.068	270.0±9.89
28 days PO	310.9±12.6	1,224.4±61.29*	3.814±0.034	3.956±0.189*	321.4±17.2*

* Significant at ($P<0.05$)