IGF-1 Modulation of Rat Cardiac Fibroblast Behavior and Gene Expression is Age-Dependent

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The collagenous extracellular matrix (ECM) forms a stress-tolerant network that is essential for proper function of the vertebrate heart. Profound changes have been detected in the interstitial ECM concurrent with developmental and disease processes of the heart. These alterations in either the organization or accumulation of ECM components markedly affect myocardial function. Studies have shown that a number of biochemical factors, including angiotensin II, transforming growth factor-β, and insulin-like growth factors, modulate collagen expression by heart fibroblasts, however, few studies have examined the differential effects of these factors on fibroblasts from animals of different physiological backgrounds. The present studies were carried out to determine whether cardiac fibroblasts isolated from different aged animals (fetal, neonatal, and adult) have diverse responses to insulin-like growth factor-1 (IGF-1). Fibroblasts isolated from fetal, neonatal, and adult rat hearts were treated with IGF-1, and several downstream responses were measured, including collagen gel contraction, adhesion to ECM, and expression of interstitial collagen and integrins. IGF-1 affected these parameters to different degrees, depending on the age of the animal from which the fibroblasts were isolated. These experiments indicate that IGF-1 is a potent modulator of fibroblast behavior in general; however, significant differences are apparent in the responsiveness of cells to this growth factor depending on the age of the animal of origin. Future experiments will be directed at determining how the *in vivo* chemical and biomechanical environment affects the response of heart fibroblasts to growth factors such as IGF-1.

Keywords. Aging, collagen, fibroblast, IGF-1

INTRODUCTION

The extracellular matrix (ECM) plays an essential role in the development and maintenance of the cardiovascular system. The composition and organization of the ECM is dynamic, particularly during periods of remodeling associated with development, aging, and disease (1, 3). Fibroblasts are the principal cell type in the heart responsible for the formation and remodeling of the ECM. Changes in several biological properties of cardiac fibroblasts including gene expression, proliferation, migration, and apoptosis may directly lead to quantitative and qualitative changes in the ECM of the heart (10, 21).

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A number of biochemical and mechanical factors have been shown to modulate the cardiac fibroblast. IGF-1, a 70-aminoacid basic polypeptide, is structurally related to proinsulin, and has a fundamental role in prenatal and postnatal cardiac development (12). In the cardiovascular system, IGF-1 increases cardiac DNA and protein synthesis, reduces protein degradation, and stimulates early neonatal cardiomyocyte proliferation and maturation (16, 17). In neonatal cardiac fibroblasts, IGF-1 stimulates proliferation and collagen synthesis (19, 23), however, the effects of IGF-1 on cardiac fibroblasts from animals of different developmental stages or physiological conditions have not been extensively studied.

Toward this goal, isolated fetal, neonatal, and adult rat cardiac fibroblasts were tested for their ability to respond to IGF-1 in bioassays involving cell-ECM interactions, including cell attachment, collagen gel contraction, and changes in gene expression. Together, these studies indicate that IGF-1 is a potent modulator of cardiac fibroblast function whose effects are dependent on the physiological background of the cell.

MATERIALS AND METHODS

Cell Isolation and Culture

Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in an AALAC-approved facility. All experiments were approved by the University of South Carolina Institutional Animal Use and Care Committee and were performed following the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH). Animals were provided food and water ad libitum. For isolation of fetal heart fibroblasts, timed-pregnant females were sacrificed at 18 days of gestation by cervical dislocation while under ether anesthesia. Fetuses were dissected from the uterus and hearts removed. Hearts were minced and digested in collagenase (4, 27). Fibroblasts were purified by selective attachment to culture dishes.

For isolation of neonatal heart fibroblasts, 5-day-old neonatal pups were sacrificed by decapitation and hearts removed. Heart tissue was minced and fibroblasts obtained as described above for fetal animals.

For isolation of adult heart fibroblasts, 8-week-old Sprague Dawley rats were sacrificed by cervical dislocation while under ether anesthesia. Hearts were removed and ventricular myocardium was minced into small pieces. Fibroblasts were obtained from explants of the myocardial tissue pieces as previously described (27).

Fetal, neonatal, and adult fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 5% fetal calf serum. At approximately 75% confluency, fibroblasts were passaged following detachment with a 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) solution. All experiments in the present study were performed with fibroblasts of passage three. Previous studies have indicated that fibroblasts *in vitro* maintain characteristics reflective of their in vivo phenotype at least to passage six (6, 18). Prior to treatment with IGF-1, fibroblasts were rinsed twice with Moscona's saline solution and cultured 24 h in serum-free media (DMEM-F12; Sigma) supplemented with 50 μg/ml ascorbic acid and 50 μg/ml glutamine.

Collagen Gel Contraction

Cardiac fibroblasts were cultured and serum-starved as described above. Fibroblasts were detached, rinsed, and resuspended at a final concentration of 400,000 cells per milliliter in DMEM-F12. Fibroblasts were mixed with an equal volume of a solution containing 1.25 mg/ml collagen type I (Cohesion Technologies, Inc., Palo Alto, CA) as previously described (5, 8). One milliliter of solution containing a final concentration of 200,000 cells per milliliter was added to bovine serum albumin-coated wells of a 24-well plate. Collagen was allowed to polymerize for one h at 37°C. Following polymerization, additional medium containing IGF-1 or vehicle was

added and the collagen gels dislodged from the sides of the wells allowing the gels to float in the media. Photographs were taken 48 h later and the area of the top surface of the gels determined as a measure of collagen gel contraction (5). Collagen gel experiments were repeated in triplicate with three different preparations of fetal, neonatal, and adult heart fibroblasts. Data are presented as the mean +/− standard deviation. Statistical analysis was performed with a Student's t-test comparison between the IGF-1-treated and the untreated samples from the same-aged animals.

Integrin-mediated cell-collagen interactions have been shown to play important roles in collagen remodeling and collagen gel contraction (5, 8). To determine the role of integrins in IGF-1 stimulated collagen gel contraction, fibroblasts were incubated in function-blocking $\beta 1$ integrin IgGs (27), for one h at 37°C. Fibroblasts were subsequently cultured in 3-dimensional collagen gels as described above. Following 48 h of culture in the presence of varying concentrations of $\beta 1$ integrin antibodies (0–80 μg/ml), contraction of the collagen gels was measured.

Cell Adhesion Assays

Fetal, neonatal, and adult cardiac fibroblasts were cultured and serum-starved as described above. Cells were treated for 24 h with 100 ng/ml IGF-1 or vehicle (sterile water). Following treatment, fibroblasts were detached by incubation in 0.25% trypsin/0.1% EDTA, rinsed, and resuspended in DMEM-F12 at a final concentration of 400,000 cells per milliliter. Cells were plated onto plasticware precoated with bovine serum albumin, collagen type I, laminin, or fibronectin as previously described (27). Fibroblasts were allowed to adhere for one h, rinsed extensively, and the adherent cells counted as previously described (19).

Western Blots

To examine the effects of IGF-1 on total cellular levels of specific integrins, fibroblasts were serum-starved then treated with varying concentrations of IGF-1 as described above. Following treatment for 24 h, fibroblasts were 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. Following extraction, total protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). 50 μg of total protein from each sample was separated on 5–15% gradient minipolyacrylamide gels. Proteins were transferred overnight to nitrocellulose at 14 Volts. An identical gel of each set of samples was run and stained with Coomassie Brilliant Blue to verify equal loading of the samples. Following transfer, the nitrocellulose membranes were rinsed briefly in Tris-Buffered Saline containing 0.1% TWEEN 20 (TBS-T). The membranes were then blocked in TBS-T containing 5% powdered milk for 1 h. Membranes were then incubated in primary antisera against the $\alpha 1$ (Chemicon, Temecula, CA) or $\beta 1$ (27) integrin chains for 2 h (19). Membranes were rinsed several times in TBS-T and incubated for 1 h in horse radish peroxidase-conjugated secondary antisera. Membranes were rinsed extensively and developed by incubation in ECL Detection Reagent (Amersham, Piscataway, NJ). Membranes were exposed to BioMax x-ray film and immunoreactive proteins subsequently quantified using an Alpha Innotech gel capture system.

Immunoprecipitation

To examine relative levels of cell surface integrins, fibroblasts were surface-labeled with biotin (EZ-Link Sulfo-NHS-Biotin, Pierce) following 24 h incubation in DMEM-F12 with or without IGF-1. Briefly, cells were incubated with or without IGF-1, and rinsed extensively in phosphate-buffered saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Fibroblasts were biotin labeled according to the manufacturer's protocol for 15 min at 4°C. Cells were rinsed in PBS containing 50 mM NH_4Cl to remove unincorporated biotin. The labeled cells were extracted in buffer containing 0.1% sodium dodecyl sulfate, 1% triton X-100, 1% deoxycholate, 0.65 mM $MgSO_4$, 1.22 mM

$CaCl_2$, 20 mM Tris-HCl (pH 7.4), and protease inhibitors. Insoluble material was removed by centrifugation and the protein concentration of the supernatant determined (BCA Protein Assay, Pierce). Equal amounts of protein from IGF-1 treated or untreated samples were then used for immunoprecipitation with specific integrin antisera. Protein extracts were incubated overnight at 4°C in primary antisera, rinsed extensively in phosphate-buffered saline, and incubated several h in protein G-sepharose. The immunoprecipitated material was rinsed again with phosphate-buffered saline, resuspended in sodium dodecyl sulfate sample buffer and separated on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and biotinylated proteins detected with the ECL kit (Amersham) following incubation with horseradish peroxidase-conjugated streptavidin.

Northern Blots

Fibroblasts were cultured and treated as described above. Following 24 h of incubation with or without IGF-1, fibroblasts were extracted in RNA-STAT60 (TElTest, Inc., Friendswood, TX), and 5 μg of total RNA was separated on 1.1% agarose gels containing formaldehyde. RNA was transferred overnight to Duralon membranes (Stratagene, La Jolla, CA). Northern blots were performed with ^{32}P-labeled cDNA probes to collagen type I (24). Blots were subsequently reprobed with a glyceraldehyde 3-phosphate dehydrogenase probe for normalization purposes. Blots were exposed for varying amounts of time to X-Omat film (Kodak). Relative quantification of mRNA was performed by Image 1 analysis of x-ray films.

Statistical Analyses

All experiments were repeated with at least three different isolations of fetal, neonatal, and adult fibroblasts. Data are presented in most of the assays as the effects of IGF-1 relative to the untreated controls. For these presentations, the untreated controls were set to 100%. All statistical analyses were made by t-test comparison between each treated condition and the corresponding untreated control.

RESULTS

Effects of IGF-1 on Heart Fibroblast Behavior

Dynamic interactions between cells and the ECM are essential in the regulation of cellular processes including migration, proliferation, and differentiation. A number of biochemical factors are known to modulate these interactions. The three-dimensional collagen model has been used extensively to assay the relative ability of cells to migrate in and remodel the ECM. Previous experiments have shown that IGF-1 stimulates contraction of collagen gels by neonatal heart fibroblasts (19). Experiments were carried out to determine if the effects of IGF-1 on collagen gel contraction are dependent on the origin of the heart cells. Fibroblasts isolated from fetal and adult hearts were cultured in collagen gels in the presence or absence of IGF-1 (100 ng/ml). Figure 1

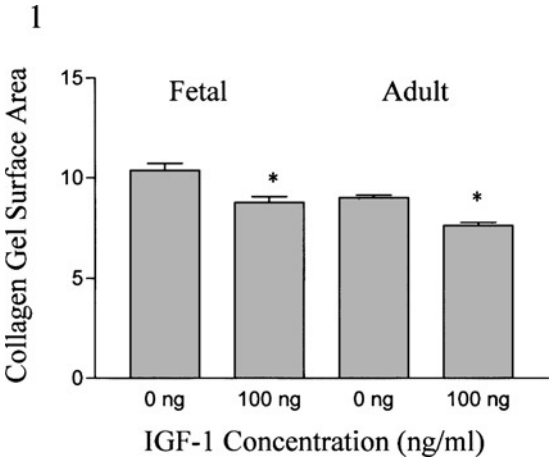

Figure 1. Effects of IGF-1 (100 ng/ml) on collagen gel contraction by fetal and adult heart fibroblasts. IGF-1 significantly stimulated collagen gel contraction with both cell types as indicated by measurements of the surface area of collagen gels (mm^2). Gels were repeated in triplicate with each of three different fibroblast isolations ($n = 9$). Data are presented as the mean $+/-$ standard deviation of the combined measurements. Significance (*) was determined by t-test analysis of corresponding treated and untreated samples.

illustrates that, similar to previous data with neonatal heart fibroblasts, IGF-1 stimulates collagen gel contraction by fetal and adult heart fibroblasts.

Experiments were also performed to examine the effects of IGF-1 on fetal, neonatal, and adult cardiac fibroblast adhesion to various ECM components. Treatment of adult heart fibroblasts with IGF-1 resulted in a substantial increase in attachment to collagen type I, laminin, and fibronectin (Figure 2c). IGF-1 had less of a stimulatory effect on adhesion of fetal fibroblasts to these ECM substrates (Figure 2a). In contrast to the results with fetal and adult heart fibroblasts, IGF-1 treatment resulted in slightly reduced adhesion of neonatal heart fibroblasts to all of the ECM components tested (Figure 2b). However, the attachment of the neonatal cells to the ECM was generally greater than the other cell types under basal (non-IGF-1) conditions.

Effects of IGF-1 on Fibroblast Gene Expression

A number of chemical factors including angiotensin II, transforming growth factor-β, and others have been identified that modulate expression of ECM components. However, the differential effects that these growth factors have at various stages of development or disease are not clear. Fibroblasts isolated from different aged animals were used to determine whether IGF-1 has different effects on gene expression dependent on the phenotype of the animal. Fibroblasts isolated from fetal, neonatal, and adult rat hearts were treated for 24 h with varying doses of IGF-1 (0–100 ng/ml), RNA extracted and Northern blots carried out with a collagen 1 (I) cDNA probe. IGF-1 treatment resulted in increased collagen type I mRNA levels by 24 h in both fetal and neonatal heart fibroblasts (Figures 3a and 3b, respectively). Contrary to this, IGF-1 had little effect on collagen mRNA levels in normal adult heart fibroblasts (Figure 3c). Similar results were obtained examining collagen type I protein levels in conditioned medium from these fibroblasts (not shown).

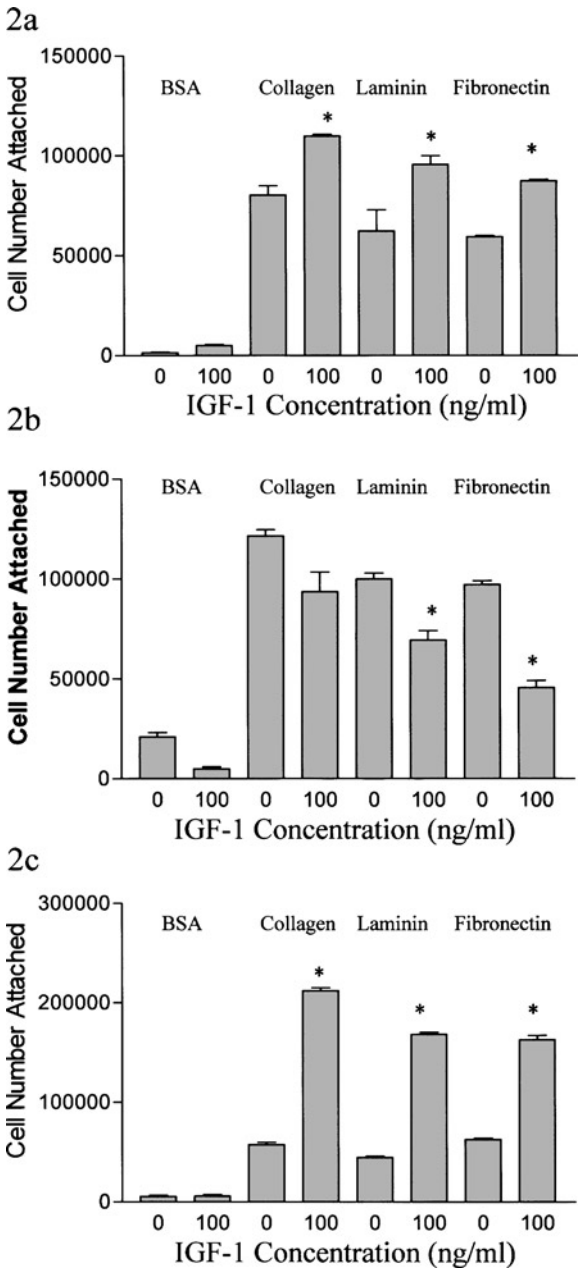

Figure 2. Attachment assays with fetal (a), neonatal (b), and adult (c) rat heart fibroblasts incubated in the absence ("0" on the X-axis of graphs) or presence ("100" on the X-axis of graphs) of IGF-1. Assays were performed with three different isolations of each cell type. Data is presented as the mean +/− standard deviation of the combined measurements. Statistical significance was determined by t-test comparison of the corresponding IGF-1 treated and untreated samples.

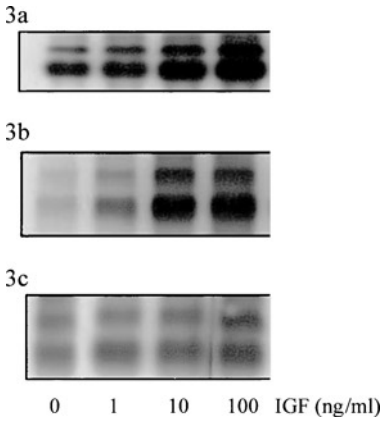

Figure 3. Representative Northern blots with an $\alpha 1$(I) collagen cDNA probe and RNA from fetal (a), neonatal (b), and adult (c) heart fibroblasts treated for 24 h with varying concentrations of IGF-1. The two bands in each figure illustrate the two mRNA transcripts of $\alpha 1$(I) collagen. These are representative of three Northern blots performed for type I collagen with each fibroblast type. Blots were reprobed with a glyceraldehyde 3-phosphate dehydrogenase cDNA to ensure even loading of RNA (not shown).

Integrins are the major family of receptors for ECM components expressed by heart fibroblasts. Experiments were carried out in the present studies to determine the effects of IGF-1 on integrin expression in fibroblasts from different aged animals. Fibroblasts were cultured in the presence of varying doses of IGF-1 for 24 h and Western blots performed to examine relative cellular levels of the $\alpha 1$ and $\beta 1$ integrins. Alternatively, following IGF-1 treatment, cell surface proteins were biotinylated, and cell surface integrin levels determined by immunoprecipitation with integrin-specific antisera. IGF-1 treatment did not significantly affect the total $\beta 1$ integrin levels in either of the fibroblast types examined (Figures 4a, 4c, and 4e). However, 24-h treatment of fetal and adult fibroblasts with IGF-1 resulted in an increase in the cell surface level of $\beta 1$ integrin protein (Figures 4b and 4f). In the fetal fibroblasts, this increase persisted at least through 48 h, while $\beta 1$ integrin levels declined back to normal levels by 48 h in the adult cells (not shown). Contrary to this, IGF-1 had no significant effects on cell surface $\beta 1$ integrin levels in neonatal heart fibroblasts (Figure 4d).

A number of different α chains can associate with the $\beta 1$ chain to form a functional integrin heterodimer in heart fibroblasts. The major collagen-binding integrins in heart fibroblasts are the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ complexes. Experiments were carried out to determine the effects of IGF-1 on the expression of the $\alpha 1$ integrin as it has previously been shown to be important in collagen gel contraction by heart fibroblasts (8). Incubation of fetal and adult heart fibroblasts with IGF-1 resulted in an increase in the total cellular level (Figures 5a and 5e) and cell surface level of $\alpha 1$ integrin protein (Figures 5b and 5f). Similar to previously-reported results (19), very little change in $\alpha 1$ integrin expression was observed in neonatal fibroblasts in response to IGF-1 treatment (Figures 5c and 5d).

Role of $\beta 1$ Integrin in IGF-1-Stimulated Collagen Gel Contraction

The above functional and protein expression data suggest that enhanced collagen gel contraction in response to IGF-1 is, at least in part, due to increased integrins on the surface of heart fibroblasts. Experiments were performed to determine whether function-blocking antibodies to the $\beta 1$ integrin chain could inhibit IGF-1-stimulated collagen gel contraction. Experiments with neonatal and adult heart fibroblasts indicated that blocking $\beta 1$ integrin function attenuates the stimulatory effects of IGF-1 on collagen gel contraction by these cell types (Figure 6). These experiments illustrated that as little as 5 μg/ml of $\beta 1$ integrin IgGs has an inhibitory effect on IGF-1-stimulated collagen gel contraction by adult heart fibroblasts (Figure 6b). The stimulatory effects of IGF-1 are completely abolished in the presence of 20 μg/ml anti-$\beta 1$ integrin. Interestingly, a much higher concentration of the $\beta 1$ integrin IgGs is required to completely block the effects of IGF-1 on collagen gel contraction by neonatal heart fibroblasts (Figure 6a). While IGF-1 does not appear to affect the cell surface levels of $\beta 1$ integrins in neonatal fibroblasts (see Figure 4), there does appear to be a higher level of $\beta 1$ integrins in neonatal fibroblasts compared to adult heart fibroblasts prior to any stimulation.

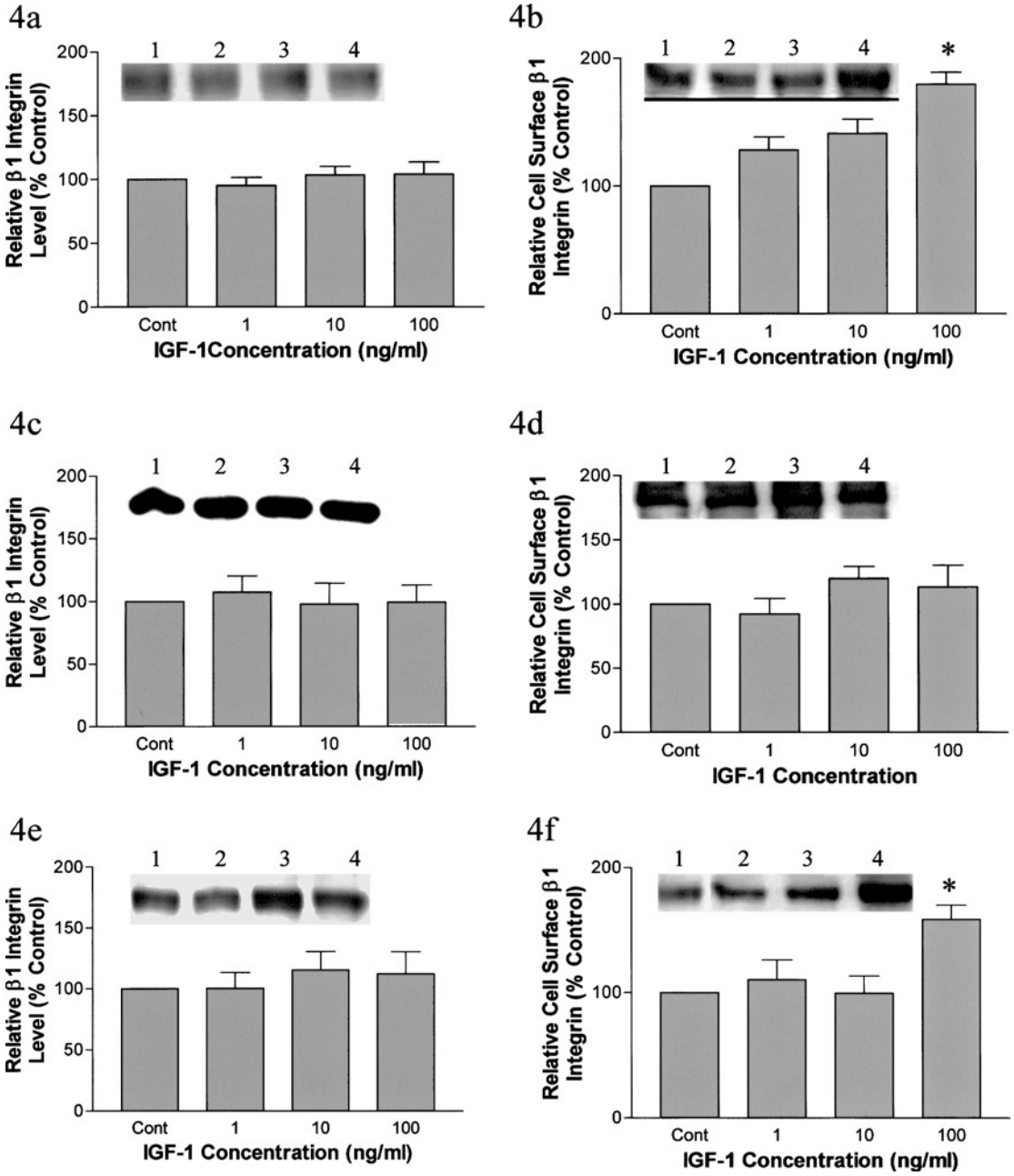

Figure 4. Analyses and representative Western blots (a, c, and e) and immunoprecipitations (b, d, and f) with β1 integrin antiserum of fetal (a, b), neonatal (c, d), and adult (e, f) heart fibroblasts treated for 24 h with varying doses of IGF-1. Western blots and immunoprecipitations were repeated with three different cell isolations. Statistical comparisons were made between each IGF-1 dose and the untreated controls. Statistical significance (*) was determined as $p < 0.05$. The lanes of the Western blots and immunoprecipitations (insets) illustrate untreated control (lane 1), 1 ng/ml IGF-1 (lane 2), 10 ng/ml IGF-1 (lane 3), and 100 ng/ml IGF-1 (lane 4) treated fibroblasts.

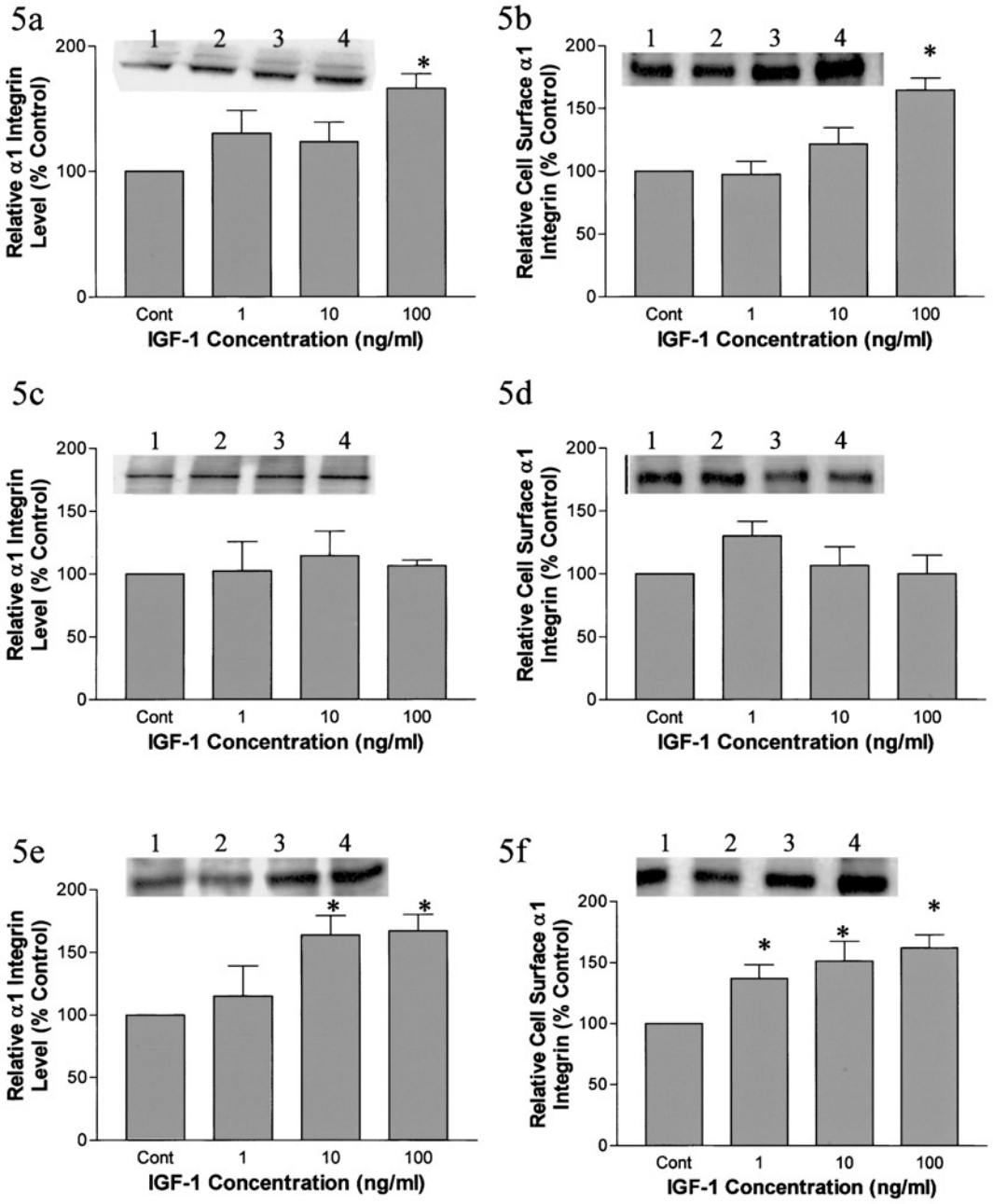

Figure 5. Analyses and representative Western blots (a, c, and e) and immunoprecipitations (b, d, and f) with an $\alpha 1$ integrin antiserum of fetal (a, b), neonatal (c, d), and adult (e, f) heart fibroblasts treated for 24 h with varying doses of IGF-1. Western blots and immunoprecipitation experiments were repeated with three different cell isolations. Statistical comparisons were made between each IGF-1 dose and the untreated controls. Statistical significance (*) was determined as $p < 0.05$. The lanes of the Western blots and immunoprecipitations (insets) illustrate untreated control (lane 1), 1 ng/ml IGF-1 (lane 2), 10 ng/ml IGF-1 (lane 3), and 100 ng/ml IGF-1 (lane 4) treated fibroblasts.

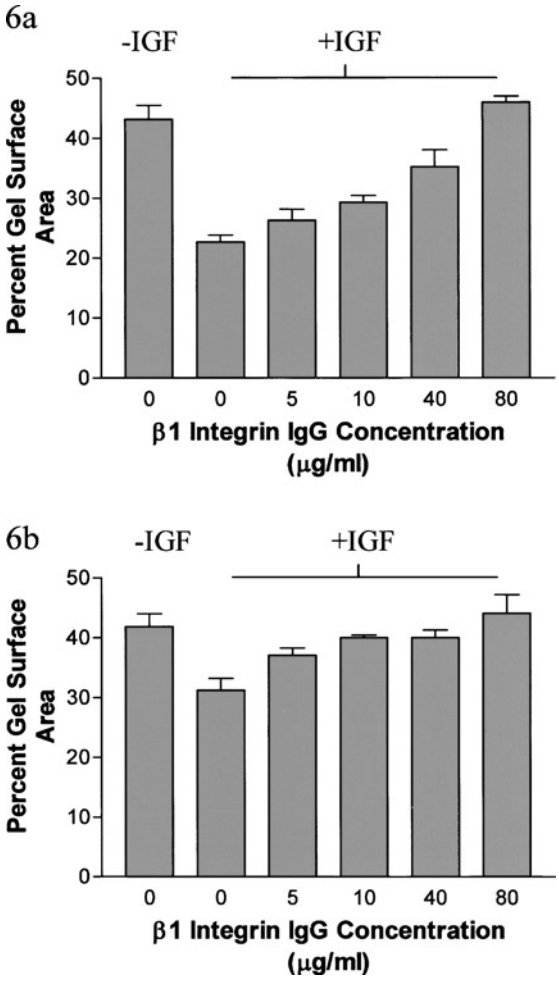

Figure 6. Analyses of the relative contraction of collagen gels by neonatal (a) and adult (b) heart fibroblasts incubated in varying concentrations of anti-β1 integrin IgGs. Collagen gels were repeated in triplicate with each condition and contraction measured relative to the initial size of the collagen gel.

DISCUSSION

The interstitial matrix of the ventricular myocardium is composed predominantly of type I and type III collagens. The collagens are organized into a specialized weave network, which, in the working myocardium, provides support for cardiac myocytes, prevents cardiac myocyte slippage, and maintains myocyte alignment during the cardiac cycle (2, 29). Historically, the collagen network has been perceived as a rather static structure playing mostly a passive role in myocardial function. It is now clear that collagen content and organization are quite dynamic, particularly during periods of development or disease. Furthermore, interactions between cells and the ECM are dynamic as well and are known to modulate cellular events including migration, proliferation, and differentiation in the heart.

A number of biochemical factors have been documented to alter either the cardiac ECM itself or interactions of heart cells with the ECM (3, 7, 9, 13, 28). Transforming growth factor-β, angiotensin II, IGF-1, and other factors stimulate collagen production by fibroblasts (11, 12, 21). Interestingly, several factors, including tumor necrosis factor-α, have been reported to be fibrotic or antifibrotic under different conditions (14, 15, 26). It is likely that fibroblasts of different origins have diverse responses to the same biochemical factors and this may explain some of the discrepancies in the literature regarding modulation of ECM expression. Experiments reported here illustrate that fibroblasts from fetal, neonatal, and adult animals have distinct responses to IGF-1 with regards to collagen gene expression. IGF-1 stimulated collagen expression in fetal and neonatal but not adult heart fibroblasts. This suggests that the same biochemical factor may have diverse effects on the cardiac fibroblasts at different stages of development or disease.

The differential response of heart fibroblasts to IGF-1 is consistent with previous work examining the wound healing properties of dermal fibroblasts (10). These studies illustrated a differential response of fibroblasts from different aged animals to growth factors that promote wound healing. The underlying mechanisms of these differential responses are currently unclear. With regards to the present studies, the physiology of the fetal, neonatal, and adult hearts are vastly different. It is possible that cardiovascular load plays an important role in the responsiveness of heart fibroblasts to specific biochemical factors. This is consistent with data from the musculoskeletal system where critical interactions have been described between mechanical and biochemical factors (20, 22). How changes in the mechanical

environment would alter the fibroblast's response to IGF-1 is unknown, but could involve alterations in receptor levels or receptor-induced signaling.

Results presented here illustrate that IGF-1 stimulates adhesion of fetal and adult, but not neonatal, heart fibroblasts. Data presented here and previous work (19) illustrate that IGF-1 treatment stimulates contraction of collagen gels by all three of these fibroblast types. Several studies have illustrated roles for collagen-binding integrins in migration within and contraction of three-dimensional collagen gels (8, 25). The present studies illustrated that the $\alpha 1\beta 1$ heterodimer, a collagen-binding integrin, is increased in fetal and adult fibroblasts in response to IGF-1. However, data presented here and previous work from this lab, indicate that IGF-1 has no effect on $\alpha 1$ integrin expression in neonatal heart fibroblasts. Together, these data suggest that increased $\alpha 1$ integrin levels in adult and fetal fibroblasts may correlate with increased adhesion, but that other mechanisms are likely responsible for changes in collagen contraction and collagen gene expression. Interestingly, cell surface levels of $\alpha 1$ integrin were increased in response to IGF-1 treatment with a corresponding increase in the total cellular levels of this protein. Contrary to this, the total cellular levels of the $\beta 1$ integrin protein remained unchanged while the cell surface levels increased in response to IGF-1. This suggests that multiple mechanisms exist for regulation of cell surface levels of individual integrin proteins.

In summary, the present studies indicate that IGF-1 has potent effects on heart fibroblast behavior and gene expression and that these effects are dependent on the age of the animal from which the fibroblasts are derived. In light of previous studies illustrating that isolated heart fibroblasts maintain their *in vivo* phenotype in culture (6, 18), we speculate that the physiological background of the cell plays a determining role in its responsiveness to IGF-1 and other biochemical factors. Further studies are required to determine how the biomechanical milieu or other parameters of the cell modulates it response to specific growth factors.

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