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Age-Dependent Expression of Collagen Receptors and Deformation of Type I Collagen Substrates by Rat Cardiac Fibroblasts

Christopher G. Wilson^{1,†}, John W. Stone^{2,‡}, Vennece Fowlkes¹, Mary O. Morales¹, Catherine J. Murphy³, Sarah C. Baxter⁴, and Edie C. Goldsmith^{1,*}

¹University of South Carolina School of Medicine, Department of Cell Biology & Anatomy, 6439 Garners Ferry Road, Columbia, SC 29209, USA

²University of South Carolina, Department of Chemistry & Biochemistry, 631 Sumter St., Columbia, SC 29208, USA

³University of Illinois at Urbana-Champaign, Department of Chemistry, A512 Chemical & Life Sciences Laboratory, 600 South Mathews Ave., Urbana, IL 61801, USA

⁴University of South Carolina, Department of Mechanical Engineering, 300 Main St., Columbia, SC 29208, USA

Abstract

Little is known about how age influences the ways in which cardiac fibroblasts interact with the extracellular matrix. We investigated the deformation of collagen substrates by neonatal and adult rat cardiac fibroblasts in monolayer and three-dimensional (3D) cultures, and quantified the expression of three collagen receptors [discoidin domain receptor (DDR) 1, DDR2, and $\beta 1$ integrin] and the contractile protein alpha smooth muscle actin (α -SMA) in these cells. We report that adult fibroblasts contracted 3D collagen substrates significantly less than their neonate counterparts, whereas no differences were observed in monolayer cultures. Adult cells had lower expression of $\beta 1$ integrin and α -SMA than neonate cultures, and we detected significant correlations between the expression of α -SMA and each of the collagen receptors in neonate cells but not in adult cells. Consistent with recent work demonstrating age-dependent interactions with myocytes, our results indicate that interactions between cardiac fibroblasts and the extracellular matrix change with age.

Keywords

cardiac fibroblast; extracellular matrix; collagen; integrin; discoidin domain receptor

Introduction

Cardiac fibroblasts comprise a substantial fraction of the cells in the adult heart (~30% in mice, ~60% in rats) (Grove et al., 1969; Banerjee et al., 2007) and serve important roles in developmental and pathologic remodeling of the myocardial extracellular matrix (ECM) (Baudino et al., 2006). During early postnatal development, cardiac fibroblasts synthesize a highly organized ECM composed primarily of type I collagen. In pathological situations, such as following a myocardial infarction, cardiac fibroblasts generate a collagen-dense scar that mechanically stabilizes the injured tissue. Typically the fibrotic response persists and ultimately compromises cardiac function (Weber & Brilla, 1993; Weber et al., 1994). Both adult and neonate cardiac fibroblasts express receptors for soluble factors that promote ECM biosynthesis (Sadoshima & Izumo, 1993; Villarreal et al., 1993), but it is unknown how the cells' capacity to remodel the ECM changes with age.

Cardiac fibroblasts can be distinguished from other cells in the heart by expression of discoidin domain receptor 2 (DDR2) (Goldsmith et al., 2004; Morales et al., 2005). DDR2 and its related family member DDR1 are receptor tyrosine kinases that bind fibrillar collagens and appear to operate independently of integrins (Vogel et al., 2000). The DDRs mediate renal and pulmonary fibrosis (Matsuyama et al., 2005; Flamant et al., 2006) and type I collagen colocalizes with DDR2⁺ cells in the developing heart (Goldsmith et al., 2010), suggesting that this class of receptors is important for collagen remodeling in the myocardium. Cardiac fibroblasts also express integrin receptors for type I collagen including the α_1 , α_2 , and β_1 subunits, and these integrins are essential for contraction of three-dimensional (3D) collagen substrates (Burgess et al., 1994; Carver et al., 1995). Expression of the cytoskeletal protein alpha smooth muscle actin (α -SMA) is positively correlated with fibroblast-mediated contraction of 3D collagen lattices (Arora & McCulloch, 1994; Hinz et al., 2001; Lijnen et al., 2003). Expression of collagen-binding integrins and α -SMA thus appears to be predictive for collagen remodeling behavior, whereas much less is known about the relationship between DDRs and contraction of collagen substrates.

The objectives of this study were to (1) compare the collagen contraction behavior of neonate and adult cardiac fibroblasts in monolayer and 3D culture, and (2) investigate the quantitative relationships between collagen receptors (DDR1, DDR2, and β_1 integrin) and the contractile protein α -SMA in these populations. We found that adult cardiac fibroblasts contracted a 3D collagen scaffold substantially less than neonate cardiac fibroblasts, a difference not observed in monolayer cultures. Quantitative age-dependent differences in the expression of collagen receptors and α -SMA at the mRNA and protein levels were observed in monolayer and 3D cultures. Regression analyses indicated that in adult cells expression of collagen receptors (DDR1, DDR2, and β_1 integrin) were decoupled from expression of the contractile protein α -SMA.

Materials and Methods

Isolation of Cardiac Fibroblasts

Cells were isolated from neonate (4 day old) and adult (~8–12 week old) Sprague Dawley rats as previously described (Banerjee et al., 2007) using a protocol approved by the

University of South Carolina Institutional Animal Care and Use Committee. For each independent experiment, neonatal hearts (approximately 30) were pooled for the isolation of cells, whereas each independent adult experiment was conducted using cells isolated from an individual animal. Whole hearts were aseptically dissected from the animals, minced in Krebs-Ringer bicarbonate buffer, digested with 100 U/mL collagenase, and mechanically dissociated by passing through a 14 gauge cannula. Digests were moved to T75 flasks, and fibroblasts were isolated by attachment to the flasks over 2 h. Cells were expanded through passage 3 or 4 in growth medium composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% newborn bovine serum, 5% fetal bovine serum, 100 U/mL penicillin G, 100 μ g/mL streptomycin, and 10 μ g/mL gentamicin.

Synthesis of Gold Nanorods

Gold nanorods were synthesized via a seed-mediated surfactant-driven approach (Jana et al., 2001a, 2001b). Briefly, nanoparticulate gold seeds (~4 nm diameter) were prepared by reducing 250 μ M chloroauric acid with 0.01 M sodium borohydride in the presence of 0.1 M hexadecyltrimethylammonium bromide (CTAB) in 18 M ω cm water. After 15 min, the seed solution was serially diluted three times, 10-fold each, in solutions of 250 μ M chloroauric acid/0.1 M CTAB/500 μ M ascorbic acid. The reaction proceeded overnight at room temperature, and the rod-shaped products (mean length: 376 ± 105 nm, mean width: 26 ± 5 nm) were purified and concentrated by centrifugation.

Monolayer Culture and Two-Dimensional Strain Measurements Using Nanoparticles

For experiments with monolayer cultures, two-chamber chamber slides were coated with type I collagen and gold nanorods as previously described (Stone et al., 2007). Briefly, pepsin-extracted acid-solubilized bovine type I atelocollagen (Advanced BioMatrix, Inc., San Diego, CA, USA) was neutralized by the addition of 0.2 N 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 9.0 and 10 $\times$ alpha minimal essential medium (α MEM) on ice at volumetric ratios of 8:1:1, respectively. The neutralized collagen (final concentration ~2.4 mg/mL) was added to each well of the chamber slide, excess collagen was removed from the wells, and the adsorbed collagen was allowed to polymerize for 2–24 h in a standard tissue culture incubator (37°C, 95% relative humidity, 5% CO₂). A second adsorbed layer of collagen, doped with as-prepared gold nanorods (10% v/v) was applied to each well and allowed to polymerize for 3 h in a culture incubator. The final thickness of the collagen coating was ~200 μ m. The addition of as-prepared gold nanorods does not appear to influence the polymerization of type I collagen (Wilson et al., 2009) and has been used previously by our group for generating strain maps of fibroblasts on collagen films (Stone et al., 2007). These particles efficiently scatter visible light and thus permit high contrast dark-field imaging and subsequent computation of substrate deformation by digital image correlation algorithms (Orendorff et al., 2005).

Neonate or adult cardiac fibroblasts were plated on the collagen-coated chamber slides at 10⁵ cells per well (~2.4 $\times$ 10⁴ cells/cm²) in the presence of 10 μ M CellTracker Orange (Invitrogen, Carlsbad, CA, USA) and allowed to adhere in a culture incubator. In pilot experiments we determined that 5 h was the minimum time for >90% of the cells to adhere and spread on these substrates. After 5 h the cultures were rinsed with fresh growth medium

to remove nonadhered cells, the upper chambers were removed from the slides, and the cultures were coverslipped. A single, randomly chosen field from each culture was imaged at 10 min intervals for 1 h by dark-field and epifluorescence microscopy (excitation filter 510–560 nm/emission filter 590 nm) on a Nikon E600 microscope with a 20× objective and SPOT Insight camera (Diagnostic Instruments, Sterling Heights, MI, USA) at room temperature. Two-dimensional (2D) strain maps were computed using particle tracking algorithms applied to the time series of dark-field images with Vic2D software (Correlated Solutions, Columbia, SC, USA). The reference image was captured at 5 h post-plating. In parallel with the imaging, identically prepared cultures (not imaged) were used for isolation of protein or RNA at 5, 5.5, and 6 h post-plating.

Three-Dimensional Collagen Substrate Contraction Assays

For 3D cultures, neonate or adult cardiac fibroblasts were suspended in neutralized type I collagen (prepared as described above) at 10^5 cells/mL. Cell-collagen suspensions were moved to 24-well tissue culture plates preadsorbed with 5% bovine serum albumin in DMEM and allowed to polymerize in a cell culture incubator. After 2 h, gels were detached from the wells in a 1 mL growth medium. Images of contracting gels were captured for up to 24 h with a Canon EOS Rebel digital camera, and gel areas were measured using ImageJ. Identically prepared cultures were collected at 0, 5, 6, 12, and 24 h for isolation of protein or RNA.

Western Blotting and Quantitative Polymerase Chain Reaction

Cell lysates for blotting were prepared by harvesting monolayer and 3D cultures in ice cold RIPA buffer (65 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, pH 7.4) with protease inhibitors (10 μ M PMSF, 10 μ g/mL leupeptin, 0.2 U/mL aprotinin, 50 μ M iodoacetamide, 5 μ M sodium orthovanadate, and 100 μ M sodium fluoride). Samples for RNA isolation were prepared by harvesting cultures in 0.5–1 mL Trizol. The lysates and RNA samples were snap frozen in liquid nitrogen and stored at -20°C prior to analysis.

For western blotting, lysates were thawed on ice and mechanically homogenized by brief ultrasonication. Lysates were then centrifuged at $10k \times g$ for 15 min, and the supernatants assayed for protein content by a detergent compatible colorimetric method (Bio-Rad Laboratories, Hercules, CA, USA). Equal quantities of protein were fractionated by SDS-PAGE, transferred to nitrocellulose, and blotted for DDR1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), DDR2 (Santa Cruz), β_1 integrin subunit (Santa Cruz), or α -SMA (Abcam, Cambridge, MA, USA). Each blot was stripped using Reblot Plus (Chemicon, now Millipore, Billerica, MA, USA) according to the manufacturer's instructions and probed for GAPDH (Chemicon) as a loading control. Blots were developed with a chemiluminescent substrate and subsaturation exposure to radiographic film. Films were scanned as digital images, and ImageJ software was used to quantify the integrated pixel density of bands migrating at the expected molecular weight of each target protein. For each blot and each sample, the pixel density for the target was normalized by the pixel density of GAPDH.

For analysis of RNA, samples were thawed on ice and homogenized by ultrasonication. After centrifugation, RNA was extracted with chloroform, loaded onto RNeasy mini columns (Qiagen, Valencia, CA, USA) and processed according to the manufacturer's directions with the optional on-column DNase 1 treatment. The RNA was then analyzed for quantity and quality by spectrophotometry at $\lambda = 260$ and 280 nm. 200 ng of total RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories) and then subjected to real-time polymerase chain reaction (PCR) using IQ SybrGreen Supermix (Bio-Rad Laboratories) and primers specific for DDR1 (forward: 5'-AGCCGGAATCTCTACGC-3'; reverse: 5'-CACAGGGTCACTCCGAA-3'), DDR2 (forward: 5'-ATCCA-ACTCCATCTTCTG-3'; reverse: 5'-ATTCTGACCACTGAC-TTG-3'), β_1 integrin subunit (forward: 5'-GCGATCAGG-AGAACCACAGAAG-3', reverse: 5'-ACAAATGAGCCAA-AGCCAATGC-3'), α -SMA (forward: 5'-GGAGTGATGGTT-GGAATGG-3'; reverse: 5'-ATGATGCCGTGTTCTATCG-3'), and the reference gene ARBP (forward: 5'-TAGAGGGT-GTCCGCAATG-3'; reverse: 5'-GAAGGTGTAGTCAGTC-TCC-3'). Data were analyzed by the C_t method of Pfaffl (2001), in which the threshold cycle number for detection of a target gene is determined relative to the threshold cycle number for a reference gene.

Statistics

Data were analyzed by the general linear model (GLM) using MiniTab version 11. Linear regressions of ln-transformed data were performed with SPSS version 16. Differences and regressions were considered statistically significant for $p < 0.05$.

Results

Our initial approach to examining the ECM remodeling behavior of neonate and adult cardiac fibroblasts was to quantify the deformation of a thin collagen substrate in monolayer cultures. Representative reference images of Cell-Tracker Orange-labeled cells and scattering from gold nanorods embedded in the collagen substrate are given in Figure 1A. The morphology of neonate and adult cells was similarly spread and stellate. Over 1 h of particle tracking, the peak principal strains (postive, tensile, ε_1 and negative, compressive, ε_2) reached a maximum at $\varepsilon_1 \sim 6.3\%$ and $\varepsilon_2 \sim -5.5\%$ for adults and at $\varepsilon_1 \sim 3.7\%$ and $\varepsilon_2 \sim -3.3\%$ for neonates (Fig. 1B); these differences between adult and neonate fibroblasts were not statistically significant. Similarly, the mean principal strains (averaged over the entire field of view, Fig. 1C) and the distributions of principal strain magnitudes (Fig. 1D) were similar for adult and neonate cultures. Collectively, these data indicate that deformation of collagen substrates by adult and neonate cardiac fibroblasts is indistinguishable in monolayer culture.

We examined culture time- and age-dependent changes in the adhesion/contraction apparatus during monolayer substrate deformation by quantifying the expression of mRNAs encoding collagen receptors DDR1, DDR2, and β_1 integrin subunit, and the contractile protein α -SMA. Expression of DDR1, DDR2, and β_1 were found to be significantly different for adult and neonate cardiac fibroblasts (Fig. 2A–C), with neonate cells exhibiting higher levels of expression (lower C_t) for all three receptors. Expression of DDR2 also

significantly increased with culture time in both adult and neonate cultures. Neonate cardiac fibroblasts had significantly higher expression of α -SMA than adult cells (Fig. 2D), and for both ages expression of α -SMA decreased with time in culture as substrate deformation plateaued. These results indicate that while neonates generally have higher expression of these mRNAs than the adult cells, the culture time-dependent shifts in transcription are similar between adult and neonate cardiac fibroblasts.

Analysis of expression of DDR1, DDR2, β_1 integrin, and α -SMA at the protein level also revealed similarities and differences between adult and neonate cardiac fibroblasts in monolayer cultures. Culture time was found to have significant effects on DDR1, DDR2, and β_1 integrin protein levels (Fig. 3A–C), whereas significant age-dependent differences were detected only for DDR2 and β_1 integrin. The GLM statistical analysis revealed a significant effect of an interaction (between culture time and age) on β_1 integrin, suggesting that regulation of this receptor is distinct between adult and neonate cells. Consistent with the patterns in mRNA expression, α -SMA protein levels decreased with culture time in both adult and neonate cardiac fibroblast cultures and were not significantly different from one another (Fig. 3D), likely contributing to the lack of observed differences in collagen deformation. These data describe additional differences between adult and neonate cells in monolayer culture and suggest that mRNA and protein expression are not tightly coupled in these cells, particularly for the receptor proteins.

We next investigated age-dependent differences in the deformation of 3D collagen hydrogels by cardiac fibroblasts (Fig. 4A). Culture time and cell age had significant effects on the extent of hydrogel compaction ($p < 0.05$) over 24 h (Fig. 4B). Neonate cardiac fibroblast populations consistently contracted the collagen hydrogels more than the adult populations (58% versus 35%). In contrast to the results of the monolayer deformation studies, these data clearly indicate that neonatal cells have a greater capacity to deform and/or remodel their ECM than adult cells.

Analysis of mRNAs encoding collagen receptors and α -SMA in the 3D cultures demonstrated significant differences in expression of DDR2 with culture time, in expression of β_1 integrin with culture time and cell age, and in expression of α -SMA with culture time and cell age (Fig. 5). Although the changes in DDR2 expression were not substantial, a pattern of expression in both adult and neonate cells marked by a peak C_t at ~5 h and subsequent decay (increase in expression) through 24 h is consistent with a minor role for this receptor in compaction and/or remodeling of the collagen scaffold. As in the monolayer cultures, α -SMA mRNA expression decreased with time, suggesting that as the cell populations approach an equilibrium state of substrate deformation, transcription of α -SMA decreases (independent of the age of the cells). Neonate cells exhibited lower C_t s (higher expression) than the adult cells for both β_1 integrin and α -SMA throughout the culture period, suggesting that these two proteins contributed significantly to the age-dependent difference in hydrogel compaction.

Expression of collagen receptor and α -SMA proteins generally corroborated the patterns of mRNA expression in the 3D cultures (Fig. 6). We detected significant effects of culture time on the expression of DDR1 and DDR2, independent of the age of the cells. Expression of

these proteins fluctuated substantially but essentially in synchrony, with peaks in expression at ~5 and ~12 h, and minima at ~6 and ~24 h. Both culture time and age of the cell were found to be statistically significant factors in the expression of β_1 integrin protein, and as in the monolayer cultures the interaction term was also a significant factor. In contrast to the monolayer cultures, however, neonate cardiac fibroblasts generally had higher expression of β_1 integrin than the adult populations. Expression of α -SMA protein was also higher in neonate than adult cardiac fibroblasts in the 3D cultures, further implicating these components of the contractile apparatus in the observed age-dependent differences in substrate deformation.

To investigate the basis for age-dependent differences in 3D substrate deformation, we explored the relationships between expression of DDRs, β_1 integrin, and α -SMA proteins via regression analyses. For both adult and neonate populations, we observed statistically significant ($p < 0.05$) power law relationships between DDR1 and DDR2 proteins independent of age and culture condition (Fig. 7A), suggesting a tight coupling in the regulation of these receptors. In contrast, significant regressions were detected between β_1 integrin and DDR2 for cells cultured in 3D collagen hydrogels, independent of age, but not for cells cultured in monolayer (Fig. 7B). We detected a significant regression between α -SMA and β_1 integrin in neonate 3D cultures, but not in adult 3D cultures or either adult or neonate monolayer cultures (Fig. 7C). Similarly, there was a significant correlation between α -SMA and DDR2 expression in neonate 3D cultures, but not in adult 3D cultures (Fig. 7D); significant regressions were also detected for both adult and neonate monolayer cultures. An identical pattern of correlation was observed for α -SMA and DDR1 expression (not shown). Collectively, these results underscore the distinct behavior of cells cultured in monolayer compared to 3D culture and suggest that the reduced contraction of the 3D collagen substrate by adult cardiac fibroblasts is due in part to a disruption of the correlated β_1 integrin/ α -SMA and DDR/ α -SMA expression patterns observed in neonate cells.

Discussion

A primary function of cardiac fibroblasts is to maintain the myocardial ECM, and throughout life these cells participate in the deposition, turnover, and organization of structural proteins including type I collagen. Cardiac fibroblasts engage fibrillar collagens through DDR- and integrin-class receptors, and exert stresses on the ECM through intracellular contractile machinery composed of proteins such as α -SMA. The results of this study demonstrate that adult cardiac fibroblasts contract 3D collagen substrates significantly less than neonate populations and implicate a decoupling of DDR/ α -SMA expression in the basis for this age-dependent difference.

An age-dependent difference in contraction of collagen substrates by cardiac fibroblasts was detected for 3D substrates but not for thin collagen films in monolayer cultures. This may be explained in part by the differences in cell-ECM interactions reported to exist between 2D and 3D cell cultures (Cukierman et al., 2001); monolayer culture conditions do not appear to support the assembly of *in vivo*-like cell-ECM adhesions and may thus obscure differences in collagen contraction that are readily observed in 3D cultures. We have previously reported that peak strains within cardiac fibroblast-contracted 3D collagen gels approach

−0.40 (i.e., 40% compression) (Baxter et al., 2008), whereas the peak strains in our monolayer cultures plateaued at $\pm\sim 0.10$. Age-related differences in contractility then may only emerge in the context of substrates mechanically permissive for greater deformation. Due to their adhesion to the underlying glass, the collagen films have ~ 10 -fold higher stiffness than the free-floating collagen gels, and this difference in compliance limits cell-induced deformation of the substrate. Substrate compliance, however, has also been shown to influence expression of α -SMA and α_2 and β_1 integrin subunits in fibroblasts (Arora et al., 1999) and, more generally, the morphology and phenotype of cells (Engler et al., 2004, 2006, 2008). Interestingly, in contrast to our results, Knezevic et al. (2002) reported no differences in the capacity of neonate and adult rat cardiac fibroblasts to contract type I collagen gels. Those experiments were performed with a higher cell density, a lower collagen concentration, and a different gel geometry than those in the current study, and a recent report indicates that contractile cooperativity between fibroblasts is dependent on the cell density and mechanical boundary conditions of 3D collagen gels (Fernandez & Bausch, 2009). Finally, the relatively short timecourse describing the behavior of the monolayer cultures, limited by our experimental setup, may be insufficient to detect age-dependent differences in contraction; our data suggest, however, that an equilibrium contraction of the collagen films was reached by <1 h.

Age-dependent differences in the expression of mRNAs encoding collagen receptors and α -SMA expression were detected in both monolayer and 3D cultures. Over the duration of the monolayer cultures, DDR1, DDR2, β_1 integrin, and α -SMA were expressed at higher levels in neonate fibroblasts than in adult-derived cells. Concomitant with collagen film contraction, transcription of mRNAs for α -SMA decreased and for DDR2 increased with time ($p < 0.05$) in both neonate and adult cultures. Although we did not detect a significant effect of age on DDR1 or DDR2 expression in the 3D cultures, β_1 integrin and α -SMA were again expressed higher in neonate compared with adult cells and the temporal patterns of DDR2 and α -SMA expression were similar between monolayer and 3D cultures for both neonate and adult cardiac fibroblasts. Collectively these results indicate that there are intrinsic age-dependent differences in the level of expression of these mRNAs and that the transcriptional regulation of DDR1, DDR2, β_1 integrin, and α -SMA was age independent in our experiments. Our observations of age-dependent expression patterns are consistent with those in a recent report demonstrating that cardiac fibroblasts from embryonic hearts had higher expression of mRNAs for ECM proteins and growth factors, such as fibronectin and pleiotrophin, respectively, than fibroblasts from adult hearts (Ieda et al., 2009).

Regression analyses revealed age-dependent correlations between the expression of DDR1, DDR2, β_1 integrin, and α -SMA proteins. Most strikingly, the significant correlations between β_1 integrin and α -SMA expression, between DDR2 and α -SMA expression, and between DDR1 and α -SMA expression observed in 3D neonate cultures were absent in adult 3D cultures. This decoupling of collagen receptor and α -SMA expression in the adult cells may underlie the age-dependent differences in contraction of 3D collagen substrates if coordinated expression of the collagen receptors with α -SMA is required for contraction. Transcription of α -SMA is regulated in part by integrin-associated focal adhesion kinase signaling (Chan et al., 2009), which suggests that β_1 integrin is an upstream regulator of α -

SMA in fibroblasts. A regulatory connection between either DDR1 or DDR2 and α -SMA expression has yet to be described; however, the expression of α -SMA was not affected by disruption of DDR2 in skin fibroblasts (Olaso et al., 2002). We have previously found that a DDR2-function blocking antibody significantly inhibited contraction of collagen gels by neonate cardiac fibroblasts by ~15% (M.O. Morales and E.C. Goldsmith, unpublished observations), indicating that DDR2 serves a quantitative role in substrate deformation, although smaller in nature than that previously reported for β_1 integrin (Carver et al., 1995). In addition, the DDRs are potent transcriptional regulators of matrix metalloproteinases (MMPs), including MMP2 (Olaso et al., 2001), MMP9 (Hou et al., 2002; Matsuyama et al., 2005), and MMP13 (Xu et al., 2005), which participate in the collagen degradation and cell migration required for contraction of 3D collagen gels. We did not observe significant correlations between DDR expression and collagen gel contraction (not shown), but our results support the hypothesis that coordinated expression of DDRs with α -SMA favors increased contraction of collagen substrates by cardiac fibroblasts.

Summary

This study provides insight into the dynamic nature of cardiac fibroblasts by demonstrating significant effects of age on the contraction of collagen substrates and on the expression of α -SMA and DDR- and integrin-class receptors. Adult cells have a diminished capacity to deform 3D collagen substrates compared to neonatal cells. This difference appears to be due to lower expression levels of β_1 integrin and α -SMA, but disruption of the coordinated expression of DDRs with α -SMA may also contribute. These results are significant in the context of understanding mechanisms by which cardiac fibroblasts interact with and remodel the ECM throughout postnatal life.

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References

- Arora PD, McCulloch CA. Dependence of collagen remodelling on alpha-smooth muscle actin expression by fibroblasts. *J Cell Physiol.* 1994; 159:161–175. [PubMed: 8138584]
- Arora PD, Narani N, McCulloch CA. The compliance of collagen gels regulates transforming growth factor-beta induction of alpha-smooth muscle actin in fibroblasts. *Am J Pathol.* 1999; 154:871–882. [PubMed: 10079265]
- Banerjee I, Fuseler JW, Price RL, Borg TK, Baudino TA. Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *Am J Physiol Heart Circ Physiol.* 2007; 293:H1883–H1891. [PubMed: 17604329]
- Baudino TA, Carver W, Giles W, Borg TK. Cardiac fibroblasts: Friend or foe? *Am J Physiol Heart Circ Physiol.* 2006; 291:H1015–H1026. [PubMed: 16617141]
- Baxter SC, Morales MO, Goldsmith EC. Adaptive changes in cardiac fibroblast morphology and collagen organization as a result of mechanical environment. *Cell Biochem Biophys.* 2008; 51:33–44. [PubMed: 18446277]

- Burgess ML, Carver WE, Terracio L, Wilson SP, Wilson MA, Borg TK. Integrin-mediated collagen gel contraction by cardiac fibroblasts. Effects of angiotensin II. *Circ Res.* 1994; 74:291–298. [PubMed: 8293568]
- Carver W, Molano I, Reaves TA, Borg TK, Terracio L. Role of the alpha 1 beta 1 integrin complex in collagen gel contraction in vitro by fibroblasts. *J Cell Physiol.* 1995; 165:425–437. [PubMed: 7593221]
- Chan MW, Arora PD, Bozavikov P, McCulloch CA. FAK, PIP5K1gamma and gelsolin cooperatively mediate force-induced expression of alpha-smooth muscle actin. *J Cell Sci.* 2009; 122:2769–2781. [PubMed: 19596799]
- Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science.* 2001; 294:1708–1712. [PubMed: 11721053]
- Engler A, Bacakova L, Newman C, Hategan A, Griffin M, Discher D. Substrate compliance versus ligand density in cell on gel responses. *Biophys J.* 2004; 86:617–628. [PubMed: 14695306]
- Engler AJ, Carag-Krieger C, Johnson CP, Raab M, Tang HY, Speicher DW, Sanger JW, Sanger JM, Discher DE. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: Scar-like rigidity inhibits beating. *J Cell Sci.* 2008; 121:3794–3802. [PubMed: 18957515]
- Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* 2006; 126:677–689. [PubMed: 16923388]
- Fernandez P, Bausch AR. The compaction of gels by cells: A case of collective mechanical activity. *Integr Biol (Camb).* 2009; 1:252–259. [PubMed: 20023736]
- Flamant M, Placier S, Rodenas A, Curat CA, Vogel WF, Chatziantoniou C, Dussaule JC. Discoidin domain receptor 1 null mice are protected against hypertension-induced renal disease. *J Am Soc Nephrol.* 2006; 17:3374–3381. [PubMed: 17093065]
- Goldsmith EC, Hoffman A, Morales MO, Potts JD, Price RL, McFadden A, Rice M, Borg TK. Organization of fibroblasts in the heart. *Dev Dyn.* 2004; 230:787–794. [PubMed: 15254913]
- Goldsmith EC, Zhang X, Watson J, Hastings J, Potts JD. The collagen receptor DDR2 is expressed during early cardiac development. *Anat Rec (Hoboken).* 2010; 293(5):762–769. [PubMed: 19479965]
- Grove D, Zak R, Nair KG, Aschenbrenner V. Biochemical correlates of cardiac hypertrophy. IV. Observations on the cellular organization of growth during myocardial hypertrophy in the rat. *Circ Res.* 1969; 25:473–485. [PubMed: 4242255]
- Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell.* 2001; 12:2730–2741. [PubMed: 11553712]
- Hou G, Vogel WF, Bendeck MP. Tyrosine kinase activity of discoidin domain receptor 1 is necessary for smooth muscle cell migration and matrix metalloproteinase expression. *Circ Res.* 2002; 90:1147–1149. [PubMed: 12065315]
- Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM, Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell.* 2009; 16:233–244. [PubMed: 19217425]
- Jana NR, Gearheart L, Murphy CJ. Seed-mediated growth approach for shape-controlled synthesis of spheroidal and rod-like gold nanoparticles using a surfactant template. *Adv Mater.* 2001a; 13:1389–1393.
- Jana NR, Gearheart L, Murphy CJ. Wet chemical synthesis of high aspect ratio cylindrical gold nanorods. *J Phys Chem B.* 2001b; 105:4065–4067.
- Knezevic V, Sim AJ, Borg TK, Holmes JW. Isotonic biaxial loading of fibroblast-populated collagen gels: A versatile, low-cost system for the study of mechanobiology. *Biomech Model Mechanobiol.* 2002; 1:59–67. [PubMed: 14586707]
- Lijnen P, Petrov V, Fagard R. Transforming growth factor-beta 1-mediated collagen gel contraction by cardiac fibroblasts. *J Renin Angiotensin Aldosterone Syst.* 2003; 4:113–118. [PubMed: 12806594]
- Matsuyama W, Watanabe M, Shirahama Y, Oonakahara K, Higashimoto I, Yoshimura T, Osame M, Arimura K. Activation of discoidin domain receptor 1 on CD14-positive bronchoalveolar lavage fluid cells induces chemokine production in idiopathic pulmonary fibrosis. *J Immunol.* 2005; 174:6490–6498. [PubMed: 15879152]

- Morales MO, Price RL, Goldsmith EC. Expression of discoidin domain receptor 2 (DDR2) in the developing heart. *Microsc Microanal.* 2005; 11:260–267. [PubMed: 16060979]
- Olaso E, Ikeda K, Eng FJ, Xu L, Wang LH, Lin HC, Friedman SL. DDR2 receptor promotes MMP-2-mediated proliferation and invasion by hepatic stellate cells. *J Clin Invest.* 2001; 108:1369–1378. [PubMed: 11696582]
- Olaso E, Labrador JP, Wang L, Ikeda K, Eng FJ, Klein R, Lovett DH, Lin HC, Friedman SL. Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association with transcriptional activation of matrix metalloproteinase-2. *J Biol Chem.* 2002; 277:3606–3613. [PubMed: 11723120]
- Orendorff CJ, Baxter SC, Goldsmith EC, Murphy CJ. Light scattering from gold nanorods: Tracking material deformation. *Nanotechnology.* 2005; 16:1601–2605.
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45. [PubMed: 11328886]
- Sadoshima J, Izumo S. Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ Res.* 1993; 73:413–423. [PubMed: 8348686]
- Stone JW, Sisco PN, Goldsmith EC, Baxter SC, Murphy CJ. Using gold nanorods to probe cell-induced collagen deformation. *Nano Lett.* 2007; 7:116–119. [PubMed: 17212449]
- Villarreal FJ, Kim NN, Ungab GD, Printz MP, Dillmann WH. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation.* 1993; 88:2849–2861. [PubMed: 8252698]
- Vogel W, Brakebusch C, Fässler R, Alves F, Ruggiero F, Pawson T. Discoidin domain receptor 1 is activated independently of beta (1) integrin. *J Biol Chem.* 2000; 275:5779–5784. [PubMed: 10681566]
- Weber KT, Brilla CG. Structural basis for pathologic left ventricular hypertrophy. *Clin Cardiol.* 1993; 16:II10–II14. [PubMed: 8504584]
- Weber KT, Sun Y, Tyagi SC, Cleutjens JP. Collagen network of the myocardium: Function, structural remodeling and regulatory mechanisms. *J Mol Cell Cardiol.* 1994; 26:279–292. [PubMed: 8028011]
- Wilson CG, Sisco PN, Goldsmith EC, Murphy CJ. Glycosaminoglycan-functionalized gold nanorods: Interactions with cardiac cells and type I collagen. *J Mater Chem.* 2009; 19:6332–6340.
- Xu L, Peng H, Wu D, Hu K, Goldring MB, Olsen BR, Li Y. Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. *J Biol Chem.* 2005; 280:548–555. [PubMed: 15509586]

Figure 1.

Deformation of collagen substrates in monolayer cultures of adult and neonate cardiac fibroblasts. **A: i:** Fluorescence image of CMRA-labeled adult rat cardiac fibroblasts cultured on a collagen-coated coverslip. **ii:** Dark-field image of the same field of view shown in panel **i**. Gold nanoparticles efficiently scatter light producing a yellow-green color, thus permitting high-resolution particle tracking for calculating strain fields. The diffuse gray-blue background is light scattered by the collagen film. **iii:** Overlay of fluorescence and dark-field images. **B:** Time courses of maximum principal strains (ϵ_1 and ϵ_2) generated by adult and neonate cardiac fibroblasts. **C:** Principal strains averaged over the field of view after 6 h monolayer culture (1 h of particle tracking). **D:** Histograms of principal strain magnitudes over each field of view. Data are mean $\pm$ SD (adult, $n = 6$; neonate, $n = 5$).

Figure 2. mRNA expression of collagen receptors (**A**) DDR1, (**B**) DDR2, (**C**) β_1 integrin, and (**D**) α -SMA vary with age and time in monolayer culture. Gene expression was quantified by real-time PCR; C_t indicates the difference between threshold cycle numbers for the target gene (e.g., DDR1) and the internal standard (ARBP, acidic ribosomal phosphoprotein). Data are mean $\pm$ SD from three independent experiments. Inset tables list *p* values for each term in the general linear model ANOVA. NS = $p > 0.1$. ★ denotes a significant difference ($p < 0.05$) between adult and neonate.

Figure 3.

Protein expression of collagen receptors (A) DDR1, (B) DDR2, and (C) β_1 integrin and (D) α -SMA vary with age and time in monolayer culture. Protein expression was quantified by densitometric analysis of western blots and are reported in arbitrary units of integrated pixel density. The density of each protein band was normalized by the density of the GAPDH band on the reprobed membrane. Data are mean $\pm$ SD from three independent experiments. Inset tables list p values for each term in the general linear model ANOVA. NS = $p > 0.1$.

Figure 4.

Neonate cardiac fibroblasts contracted a 3D collagen scaffold significantly more than adult cardiac fibroblasts. **A:** Representative images of cell-seeded collagen hydrogels. **B:** Contraction was quantified by measuring the area (A) over 24 h and normalizing each measurement by the initial area (A_0). Data are mean $\pm$ SD from four independent experiments. ★ denotes a significant difference ($p < 0.05$) between adult and neonate.

Figure 5. mRNA expression of collagen receptors (A) DDR1, (B) DDR2, and (C) β_1 integrin, and (D) α -SMA vary with age and time in 3D culture. Gene expression was quantified by real-time PCR; C_t indicates the difference between threshold cycle numbers for the target gene (e.g., DDR1) and the internal standard (ARBP, acidic ribosomal phosphoprotein). Data are mean $\pm$ SD from three independent experiments. Inset tables list p values for each term in the general linear model ANOVA. NS = $p > 0.1$. ★ denotes a significant difference ($p < 0.05$) between adult and neonate.

Figure 6. Protein expression of collagen receptors (A) DDR1, (B) DDR2, and (C) β_1 integrin and (D) α -SMA vary with age and time in 3D culture. Protein expression was quantified by densitometric analysis of western blots and are reported in arbitrary units of integrated pixel density. The density of each protein band was normalized by the density of the GAPDH band on the reprobed membrane. Data are mean $\pm$ SD from three independent experiments. Inset tables list *p* values for each term in the general linear model ANOVA. NS = *p* > 0.1. ★ denotes a significant difference (*p* < 0.05) between adult and neonate.

Figure 7.

Regressions of receptor and α -SMA protein expression reveal age- and culture condition-dependent correlations. Data were $\ln$ -transformed and then analyzed by linear regression. Regression curves for monolayer cultures are given by dashed lines, and regressions for 3D cultures are given by solid lines. ★ denotes a statistically significant correlation ($p < 0.05$).