Collagen Expression in Mechanically Stimulated Cardiac Fibroblasts

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The cardiac extracellular matrix, composed predominantly of collagenous fibers, forms a stress-tolerant network that facilitates the distribution of forces generated in the heart and provides for proper alignment of cardiac myocytes. Although considerable information exists regarding the morphological organization of the heart extracellular matrix, little is known about the regulation of the synthesis and accumulation of extracellular matrix components. A potentially significant factor in the cardiovascular system is mechanical stimulation including changes in physical tension and pressure. We recently have developed an in vitro model system to elucidate the effects of mechanical stretch on isolated populations of heart cells. In the present study, we have used biochemical and molecular biological techniques to analyze changes in collagen synthesis by cardiac fibroblasts in response to mechanical stretch. These studies show that the ratio of collagen type III to collagen type I increases in mechanically stretched cells. They also show that type III collagen mRNA levels are increased in response to cyclic mechanical stretch for durations as short as 12 hours. Type I collagen mRNA levels were not found to change under the stretch conditions used in this study. Our results emphasize the potential regulatory role of mechanical stimulation in the expression of specific genes in the heart and support previous studies indicating this to be an intriguing in vitro model of cardiac hypertrophy. (Circulation Research 1991;69:116–122)

The arrangement of the extracellular matrix (ECM) of the heart is intimately associated with cardiac function during development and disease (for reviews, see References 1–3). The complex three-dimensional arrangement of glycoproteins and proteoglycans forms an elastic, stress-tolerant network in response to physiological signals such as changes in pressure and volume. Although several distinct types of collagen exist in the ECM, the cardiac interstitium is composed predominantly of type I and III collagens. Previous structural and biochemical studies have shown definitively the dynamic nature of the interstitial matrix of the heart.4–6 During physiological hypertrophy of the neonatal heart5–7 and in early stages of adult pressure-overload hypertrophy, the arrangement of the ECM and, in particular, the interstitial collagens is altered.8,9 Total collagen volume also is disproportionately increased during the rapid growth of the neonatal rat heart10 and in the hypertrophied adult myocardium.4,11,12 Recently, it has been demonstrated that the relative proportions of the type III and type I collagen proteins also are altered in the hypertrophied rat myocardium.13 These changes in the organization and accumulation of the interstitial collagens can be correlated with concurrent changes in myocardial compliance.4

It has become increasingly evident that elaborate changes occur in the synthesis and organization of the interstitial collagens during periods of increased pressure and growth in the heart. These ECM components are thought to play important roles in the function of the heart, such as in the compliance of the heart wall,4 in the proper alignment of the cardiac cells,7,14 and in myofibrillogenesis.15 As such, it is important to understand how the synthesis, accumulation, and organization of the ECM components are regulated in the heart. Initial investigations have shown that the fibroblasts located within the myocardium are responsible for the synthesis of the interstitial collagens.16 Although the fibroblasts are responsible for interstitial collagen synthesis, the attachment of the collagen fibers to the surface of myocytes is an essential contribution to the three-dimensional arrangement of the ECM.17 It is clear then that the organization of the heart ECM involves the interaction of a number of cell types.

Several studies have illustrated the effects of various chemical factors on collagen synthesis, the most
studied of these being the transforming growth factor β family.18–20 However, the role that these growth factors play in the heart is not known. The physical milieu of cells, including the surrounding matrix, tension, and mechanical stretch also can influence ECM synthesis.21–23 The role of the physical environment is particularly important in the cardiovascular system, in which cells are continually subjected to changes in pressure and volume. We recently have developed an in vitro system to analyze the effect of linear stretch on isolated populations of cardiac cells.24–26 Initial studies suggest that mechanical stretch is sufficient to induce hypertrophy in isolated heart myocytes and to stimulate morphological alterations in the cell and its cytoskeleton.

In the present study, we subjected isolated neonatal rat heart fibroblasts, which produce the interstitial collagens16,27 to cyclic or static (passive) mechanical stretch to 1) examine the effects of linear stretch on the synthesis of these ECM components, and 2) further evaluate the applicability of this system in the study of cardiac hypertrophy where known changes in collagen synthesis and accumulation occur. These experiments demonstrate that mechanical stretch stimulates type III collagen protein and mRNA synthetic rates in cyclically stretched fibroblasts. These results are similar to the events that occur in early stages of cardiac hypertrophy in vivo.

Materials and Methods

Cell Culture and Mechanical Stretch

All animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care, and all protocols were approved by the Institutional Animal Review Board. Neonatal Sprague-Dawley rats were decapitated, the heads were placed in liquid nitrogen, and the hearts were placed in ice-cold phosphate buffered saline. Fibroblasts were isolated28,29 and, after several passages (less than four), plated on laminin-coated silicone elastomer (Silastic) membranes (Dow Corning, Midland, Mich.) as previously described.24 Cells were cultured in Dulbecco’s modified Eagle medium with 5% fetal bovine serum and 10% newborn bovine serum. Fibroblasts were allowed to adapt to culture and attach to the substrates for 5–7 days. Membranes to be stretched were placed into either an apparatus designed to induce cyclic linear stretch or an apparatus designed to induce passive linear stretch.24 Substrates were stretched at a rate of 20 cycles per minute and 5% linear stretch for varying durations of time. Static stretches were at 5% linear stretch for identical durations of time. Increased percentages of stretch also were examined; however, this resulted in the loss of large numbers of fibroblasts from the membranes. Control cells that were not stretched also were cultured on laminin-coated membranes for the same time periods as stretched cells.

Collagen Protein Synthesis

To analyze relative rates of collagen synthesis, cells were cultured in the presence of [3H]proline, [3H]-glycine (10 μCi/ml), or both with sodium ascorbate (50 μg/ml) and glutamine (300 μg/ml) for the duration of the stretch (24 hours). Medium was collected from the cell cultures and precipitated with 40% saturated ammonium sulfate. The precipitate was resuspended on 0.5N acetic acid and pepticin (2 mg/ml) digested at 4°C for 12 hours. The soluble fraction was dialyzed against several changes of 0.02 M phosphate buffer (pH 9.0). The collagensous precipitate was resuspended in 0.1% sodium dodecyl sulfate. The type I and type III collagen chains were separated by gel electrophoresis using thiohlycronic acid as the reducing agent.30 Gels were fixed in 50% methanol/5% acetic acid, rinsed in water, then soaked in Enlightening (Du Pont Co., Wilmington, Del.) 30–45 minutes. Gels were rinsed briefly in water, dried, and exposed to X-Omat x-ray film (Eastman Kodak Co., Rochester, N.Y.) for 4–6 weeks at −70°C. Type I and III collagen α chains were identified by comparison to collagen I and III standards, by immunoblots with a type I collagen antiserum, and by running identical gels in the absence of the reducing agent.30 Autoradiographs were scanned with a densitometer (Pharmacia LKB Biotechnology, Piscataway, N.J.) using the GSXL scan program. Several exposure times
were scanned for each experiment. After laser scanning, the collagen α chains were cut from the gels, and radioactivity in each was quantitated by liquid scintillation counting. This served to confirm relative quantitation by laser scanning.

Northern and Dot Blot Analysis

Total cellular RNA was extracted from fibroblasts by the guanidine thiocyanate method.\textsuperscript{31} Equal amounts of RNA (determined spectrophotometrically) were size-fractionated by electrophoresis on 1.5% agarose gels containing formaldehyde.\textsuperscript{32} After electrophoresis, RNA was transferred to Hybond N membranes (Amersham Corp., Arlington Heights, Ill.) by capillary blots according to the manufacturer's protocol. Membranes were baked for 2 hours at 80°C and prehybridized in 5× standard saline phosphate ethylenediamine/tetraacetic acid buffer (SSPE) (20× SSPE is 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA) and 50% formamide at 42°C. Hybridization was carried out in 5× SSPE, 50% formamide, 10% dextran sulfate, and 2×10\textsuperscript{6} cpm \textsuperscript{32}P-labeled probe per milliliter hybridization buffer at 42°C overnight. Subsequently, the blots were washed in 0.2× standard saline citrate buffer (SSC) (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 65°C, and autoradiography was carried out at −70°C. Blots were scanned using a Pharmacia LKB densitometer as described above. Hybridization signals with collagen cDNAs were standardized by comparison to signals of identical blots with an actin cDNA found not to be altered with mechanical stretch.

For slot blot analysis, 5- and 10-μg aliquots of each RNA sample were denatured with formaldehyde and vacuum blotted to Hybond N membranes according to the manufacturer's protocol (Amersham). Blots were processed and analyzed as described above.

\textbf{FIGURE 2.} Graphic representation of the relative ratios of type III/type I collagen synthesis in nonstretched (NON), static stretched (STAT), and cyclic stretched (CYC) fibroblasts.

\textbf{In Situ Hybridization}

Cells were fixed for in situ hybridization in 2% paraformaldehyde for 30 minutes, rinsed in phosphate-buffered saline several times, and stored in 70% ethanol at 4°C. Silastic membranes containing cells were rehydrated and digested in 5 μg/ml proteinase K at 37°C for 30 minutes. Cells were rinsed in 0.1 M triethanolamine and acetylated\textsuperscript{33} for 10 minutes. Membranes then were rinsed several times in 2× SSC and dehydrated. Cells then were hybridized to type I or III collagen antisense riboprobes in 50% formamide, 2× SSC, tRNA, and 2×10\textsuperscript{6} cpm \textsuperscript{35}S-labeled probe for 16 hours at 60°C. After hybridization, cells were rinsed several times in 4× SSC at room temperature followed by 0.1× SSC at 65°C. Cells then were digested with RNase A (50 μg/ml) at 37°C for 30 minutes and rinsed several more times in 0.1× SSC at 65°C. Membranes then were dehydrated, attached to microscope slides, and dipped in Ilford K.5D emulsion (Polysciences Inc., Piscataway, N.J.). After exposure at 4°C for 3 days to 2 weeks, slides were developed, stained with hematoxylin and eosin, and examined. Membranes processed under the above conditions except hybridized to sense probes were used as controls. Silver grains were counted and analyzed using an Image I/AT Digital image analysis system (Universal Imaging Corp., West Chester, Pa.).

\textbf{FIGURE 3.} Northern blots with cDNAs specific for α1(I) (panel A), α1(II) (panel B), and α1(III) (panel C) collagen mRNA. Each blot shows mRNA from nonstretched controls (lane 1), static (lane 2), and cyclic (lane 3) stretched fibroblasts. mRNA transcript sizes were calculated from size markers.
Membranes processed within the same experiment were used for quantitative analysis to ensure that conditions were identical for each membrane. For quantitation, the number of grains from regions outside of cells was taken as background and subtracted from grains over cells. The data were analyzed using one-way analysis of variance. Statistical significance was taken as a value of \( p < 0.05 \).

**Nucleic Acid Probes**

Plasmids pHCAL1U, pHCAL2, and pHFS3 containing cDNA inserts for procollagens \( \alpha_1(I) \), \( \alpha_2(I) \), and \( \alpha_1(III) \), respectively, were obtained from Dr. E. Vuorio (University of Turku, Turku, Finland). For Northern and dot blot analyses, appropriate restriction fragments \( 34,35 \) were purified by low melting gel electrophoresis and labeled by random primer extension using \( {\[^{32}\text{P}]{dCTP}} \) (3,000 Ci/mmol; Amersham). Radiolabeled probes were purified by Sephadex G-50 columns (Sigma Chemical Co., St. Louis, Mo.).

For in situ hybridization, appropriate restriction fragments were subcloned into the pSP73 vector (Promega Corp., Madison, Wis.), and antisense and sense RNA probes were generated with \( {\[^{35}\text{S}]{UTP}} \).
Transcription of probes and purification followed the manufacturer’s protocol.

**Results**

**Protein Synthesis**

Noninterrupted gel electrophoresis of tritiated proteins synthesized by cultured fibroblasts allows excellent resolution of the individual α chains of the type I and type III collagens (Figure 1). Densitometric scanning of the α(I) and α(III) chains was used to compare the relative synthetic rates of the type I and type III collagens in nonstretched, static stretched, and cyclic stretched fibroblasts (Figure 2). This illustrates that the type III/type I ratio increases by approximately 70% in the cyclic stretched cells relative to the nonstretched controls. These results also indicate that static stretched cells slightly increase the type III/type I ratio over nonstretched fibroblasts. Liquid scintillation counting of appropriate bands cut from gels reveals similar ratios as those obtained from laser scanned gels (Table 1). Analysis of stretched and control cells also shows an increase in the hydroxyproline accumulation in stretched cells, indicative of an overall increase in collagen accumulation (data not shown).

**mRNA Analysis**

Northern blot analysis of total RNA from nonstretched, static stretched, and cyclic stretched fibroblasts probed with pHCAL1U illustrates two α(I) transcripts of 6.7 and 4.8 kb (Figure 3A). Hybridization with pHCAL2 illustrates a major band of 5.4 kb and a minor species of 5.8 kb, indicative of α(III) mRNAs (Figure 3B), whereas pHFS3 hybridizes to a single transcript of 4.6 kb for α(III) mRNA (Figure 3C). Density scans of the autoradiographs illustrate that type III procollagen mRNA increases approximately twofold after 24 hours of cyclic stretch, whereas type I procollagen mRNA does not change (not shown).

In situ hybridization of fibroblasts with collagen RNA probes was used to verify that collagen expression was changing in response to 24 hours of stretch. Low magnification micrographs of in situ hybridization with the type III collagen probe illustrates that cardiac fibroblasts, like myocytes, undergo a change from a random orientation to an orientation perpendicular to the direction of stretch (Figure 4A). Higher magnification micrographs illustrate the specificity of the α(I)(III) (Figures 4C and 4D) and α(I) (Figures 4E and 4F) procollagen probes. These also illustrate the higher density of grains over cyclic stretched cells (Figure 4D) relative to nonstretched cells (Figure 4C) with the type III collagen probe. The grain distribution over nonstretched (Figure 4E) and cyclic stretched (Figure 4F) cells is very similar with the α(I) procollagen probe. Figure 4B illustrates that very few grains are seen over cells with the sense probe.

Quantitation of grains over cells after in situ hybridization with the α(I)(III) collagen probe shows an increase in mRNA levels of approximately 60% in cyclic stretched cells and a slight increase in static stretched cells relative to nonstretched controls (Figure 5). Analysis of variance indicates a significant difference at $p<0.0001$. Quantitation of grains with the α(I) probe shows no significant increase under the stretch conditions in these experiments (Figure 5).
Dot blot analysis was used to examine the temporal response of collagen expression to stretch. These data illustrate no significant increase in $\alpha_1$(I) (Figure 6) or $\alpha_2$(III) (data not shown) procollagen mRNA levels at any of the time points analyzed. This also shows that type III procollagen mRNA levels increase by 12 hours after the start of stretch (Figure 6).

Discussion

The continued development of the neonatal rat heart involves a number of biochemical and structural changes,\textsuperscript{5,6,9} including the rapid development of the myocardial collagenous network that surrounds and connects to the cardiac myocytes.\textsuperscript{6,14} Likewise, the pressure-overload hypertrophied myocardium undergoes an elaborate rearrangement of the cardiac ECM.\textsuperscript{4,8,9} These studies illustrate the dynamic nature of the cardiac ECM. Although a number of studies have described the structural and biochemical changes that occur in the developing and diseased heart, very little is known regarding the induction of these processes. A common factor in these developmental and disease states is increased pressure and mechanical tension in the heart. Although this may be important, the precise roles of chemical and physical factors in ECM synthesis and organization in the heart remain to be elucidated.

An important question is how increases in pressure or tension during development and disease result in structural rearrangement of the ECM and alterations in the expression of specific genes in heart cells. To begin to elucidate the mechanisms by which cardiac cells respond to mechanical stimulation, we have developed a model system whereby isolated cardiac cells are either cyclically or passively stretched in vitro. Previous studies have illustrated that cardiac myocytes undergo morphological reorientation and cytoskeletal reorganization in response to cyclic mechanical stretch.\textsuperscript{24,26} A number of changes also are seen indicative of cellular hypertrophy in stretched myocytes, including increased RNA synthesis, increased total protein synthesis, and increased cell size.\textsuperscript{25} However, little is known regarding the response of cardiac fibroblasts to mechanical stimulation. The results of the present study demonstrate that cyclic mechanical stretch of as little as 5% is capable of stimulating type III collagen synthesis in neonatal heart fibroblasts.

Previous studies have illustrated that an overall increase in collagen volume accompanies the structural modifications in the ECM of the hypertrophied heart.\textsuperscript{12} Furthermore, the type III/type I collagen ratio increases in the hypertrophied myocardium.\textsuperscript{13} The type III/type I ratio also is high in the young rat heart.\textsuperscript{37} These studies have shown that there exists a specific temporal pattern of expression of the interstitial collagens. In the earlier phases of collagen remodeling, type III collagen accumulation is increased relative to type I. This appears to be the case in the neonatal heart\textsuperscript{38} and in the early phase of cardiac hypertrophy.\textsuperscript{4} The results presented here agree with those from the in vivo hypertrophy studies, in that the collagen type III/type I protein synthetic rates increase in response to stretch. The data presented here also illustrate that the alterations seen in the proportions of the type I and type III collagens is at least partly regulated at the level of the collagen mRNAs.

The functional significance of changes in the relative proportions of type I and type III collagens in the interstitium has not been absolutely determined; however, several investigators have pointed out the structural differences in these collagens. Changes in the ratios of type I to type III collagen may result in altered elasticity of the heart wall. The results presented here agree with those from the in vivo hypertrophy studies, in that the collagen type III/type I protein synthetic rates and type III mRNA levels increase in response to stretch. However, using only 5% stretch for short periods of time (24 hours), we did not detect changes in type I collagen mRNA levels. Further studies are being carried out to elucidate the differences in the response of type I and type III collagen expression to stretch. These include experiments changing the duration and force of the applied stretch of the fibroblasts.

A number of studies have illustrated the effects of various chemical factors on collagen gene expression, including transforming growth factor $\beta$,\textsuperscript{19,20} glucocorticoids,\textsuperscript{37} and ascorbic acid.\textsuperscript{39} Cell density,\textsuperscript{40} growth rate,\textsuperscript{41} and ECM environment\textsuperscript{23} also have been demonstrated to influence collagen synthesis in vitro. However, few studies have looked directly at mechanical stimulation of collagen expression. The data presented here show a specific increase in type III collagen in response to cyclic stretch. It presently is not clear how mechanical stretch is transduced into increases in collagen expression; however, second-messenger systems may be involved in this process.\textsuperscript{22} It also is not known if mechanical stretch may act synergistically with any of the previously mentioned chemical factors known to influence collagen synthesis. Experiments using cells cultured in defined media will be required to examine these questions. The data presented here indicate this to be an excellent system to begin to address these questions.

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