Enhanced expression of fibrillin-1, a constituent of the myocardial extracellular matrix in fibrosis

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Bouzeghrane, Fatiha, Dieter P. Reinhardt, Tim L. Reudelhuber, and Gaétan Thibault. Enhanced expression of fibrillin-1, a constituent of the myocardial extracellular matrix in fibrosis. Am J Physiol Heart Circ Physiol 289: H982–H991, 2005. First published April 22, 2005; doi:10.1152/ajpheart.00151.2005.—Fibrillin-1 localization in the myocardium and the modulation of its expression in cardiac fibrosis were examined. In normal rat hearts, fibrillin-1 was abundant throughout the myocardium as thin fibers that crossed over the perimysium and around arteries. After cardiac fibrosis was induced in rats by either 14-day ANG II infusion or 21-day DOCA-salt treatment [a high endothelin-1 (ET-1) model], fibrillin-1 immunostaining was stronger in the interstitium (2.8-fold and 4.4-fold increases, respectively, in each model), extended between myocytes, and accumulated in microscopic scars and in the perivascular area of both ventricles. mRNA analysis confirmed its enhanced ventricular expression in both groups of rats (2.5-fold and 6.6-fold increments, respectively, in each model). In 1B normotensive and 2C hypertensive transgenic mice, two lines expressing an ANG II fusion protein in cardiac myocytes, strong fibrillin-1 immunoreactivity was observed in the interstitium and around arteries (3.7-fold and 7-fold increases, respectively, in each model). In 1B normotensive and 2C hypertensive transgenic mice, two lines expressing an ANG II fusion protein in cardiac myocytes, strong fibrillin-1 immunoreactivity was observed in the interstitium and around arteries (3.7-fold and 7-fold increases, respectively, in each model). In 1B normotensive and 2C hypertensive transgenic mice, two lines expressing an ANG II fusion protein in cardiac myocytes, strong fibrillin-1 immunoreactivity was observed in the interstitium and around arteries (3.7-fold and 7-fold increases, respectively, in each model). In 1B normotensive and 2C hypertensive transgenic mice, two lines expressing an ANG II fusion protein in cardiac myocytes, strong fibrillin-1 immunoreactivity was observed in the interstitium and around arteries (3.7-fold and 7-fold increases, respectively, in each model).

It is well known that cardiac hypertrophy, myocardial infarction, and heart failure are associated with changes in the expression of ECM proteins as well as their transmembrane receptors the integrins (20, 44). Reactive and reparative fibrosis profoundly alters the mechanical properties of the myocardium, affects myocyte metabolism and performance, and ultimately leads to left ventricular dysfunction (9, 35, 64).

However, the biological role of fibrillin-1 in the myocardium remains largely unstudied and is often related to Marfan’s syndrome. Nothing is known yet about the factors regulating normal fibrillin synthesis in processes, such as development and wound healing, and those that lead to abnormal fibrillin expression that may occur in several disorders, such as atherosclerosis and cardiac fibrosis. Fibrillin-1 being implicated in the pathogenesis of fibrosis of the liver and the reticular dermis of systemic sclerotic skin (12, 30, 33, 46), we hypothesized that it is a constituent of the myocardial interstitium, and, consequently, its expression may be affected like other ECM proteins in the development of cardiac fibrosis. We compared the cardiac distribution of fibrillin-1 with that of interstitial collagen and fibronectin in the normal cardiac interstitium and examined fibrillin-1 expression and modulation in vivo in experimental models of cardiac fibrosis in rats and mice. In addition, we conducted in vitro binding studies to determine whether fibrillin-1 fragments interact with integrins, and we investigated the potential role of ANG II and TGF-β1 in cardiac fibroblast fibrillin-1 secretion.

THE MYOCARDIAL EXTRACELLULAR MATRIX (ECM) represents a complex three-dimensional network of various macromolecules. The composition of the cardiac interstitium includes the fibrillar collagen network, basement membrane, proteoglycans and glycosaminoglycans, noncollagenous glycoproteins, growth factors, extracellular proteases, and adhesion receptors. The major ECM proteins are collagen type I (80% of total newly synthesized collagen), fibronectin, and, to a lesser extent, collagen types III, IV, V, and VI, and laminin as well as elastin (6). The composition of the matrix of the interstitium and the proportion and the expression of each protein can have a profound influence on cardiac structure and compliance that will determine its hemodynamic functions (20).

The elastic fibers in the ECM of connective tissues or artery walls are composed of elastin associated with 10- to 12-nm microfibrils. Fibrillin-1, a 350-kDa glycoprotein and a major component of microfibrils, is believed to form a template for tropoelastin deposition during elastic fibrogenesis (46). The presence of a RGD motif in the structure of fibrillin-1 has been suggested to mediate cell binding via α5β3- and αβ1-integrins (3, 41, 47). Moreover, immunofluorescence labeling has established a connection between fibrillin-1 and αβ3-integrin with focal adhesions (62). Microfibrils and fibrillins have been shown to be associated or to interact with a number of other ECM components (27). These observations indicate that fibrillin-containing microfibrils may anchor certain ECM elements to cells. Fibrillin-1 has been demonstrated to play an important role in tissue homeostasis (40), and fibrillin-containing microfibrils show elastic properties (28). Mutations of the fibrillin-1 gene cause Marfan’s syndrome, which is characterized, besides other symptoms, by weakening and aneurysms of the great artery walls (43).

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FIBRILLIN-1 EXPRESSION IN CARDIAC FIBROSIS

H983

MATERIALS AND METHODS

Animal Models

Housing and experimentation were in accordance with Canadian Council for Animal Care guidelines and were approved by the local animal care committee.

DOCA-salt hypertension. Male Sprague-Dawley rats (200 g, Charles River, St. Constant, Quebec, Canada) were right unilaterally nephrectomized. With the rats under light 3% isoflurane anesthesia (Abbott Laboratories, St. Laurent, Quebec, Canada) at an O2 flow rate of 1 l/min via a conical facemask, silicone pellets, releasing 200 mg of DOCA (Sigma-Aldrich, St. Louis, MO) over 21 days (13), were implanted subcutaneously by incision of the right flank. Anesthesia was maintained with 1% isoflurane. Some animals were given 0.171 mg/kg saline solution in tap water ad libitum (DOCA-salt group). The controls underwent right nephrectomy and received a silicone implant without DOCA and tap water to drink. After 21 days of treatment, blood pressure (BP) rose to 225 ± 9 mmHg compared with 112 ± 3 mmHg (P < 0.01) in control animals. Heart-to-body weight and heart weight-to-tibia length ratios were increased from 3.61 ± 0.28 to 4.86 ± 0.27 mg/g (P < 0.05) and 25.8 ± 1.15 to 33.1 ± 1.45 g/mm (P < 0.05), respectively.

Chronic ANG II infusion. Male Sprague-Dawley rats were infused via Alzet miniosmotic pumps (model 2002, Durect, Cupertino, CA) with ANG II (Calbiochem, La Jolla, CA) at a rate of 500 ng/min (53). In the chronic ANG II infusion, BP was significantly increased compared with vehicle-infused rats (122.1 ± 3.6 vs. 208.3 ± 5.4 mmHg, P < 0.05). Heart-to-body weight and left ventricular weight-to-body weight ratios were increased from 3.37 ± 0.12 to 4.13 ± 0.11 mg/g (P < 0.05) and 1.55 ± 0.06 to 1.88 ± 0.03 mg/g (P < 0.05), respectively.

Transgenic mice. Two mouse transgenic lines (1B and 2C) expressing an ANG II-producing fusion protein exclusively in cardiac myocytes were used at 12 wk of age (60). Expression of the transgene led to augmented cardiac ANG II levels in the heart and release of the peptide into the circulation. As reported in the 1B normotensive transgenic line, a 30-fold increase of cardiac ANG II level did not directly induce cardiac hypertrophy but did increase ventricular interstitial fibrosis. In the second transgenic line (2C), a 1,000-fold increase of cardiac ANG II level caused a spillover in circulating ANG II leading to hypertension and structural remodeling of the heart, which is characterized by ventricular concentric hypertrophy and cardiac fibrosis. These animals were thus chosen as a model of cardiac fibrosis allowing discrimination between local and systemic effects of ANG II on cardiac fibrillin-1 deposition.

The rats and mice were anesthetized, and their hearts were arrested in diastole by perfusion with PBS containing 50 mmol/l KCl via the left ventricle for 5 min. Some hearts were weighed, frozen immediately in liquid nitrogen, and stored until further processing for biochemical study. Other hearts were fixed overnight in Zn-Tris solution (4), dehydrated, and embedded in paraffin by a routine histological procedure for immunohistochemistry.

Histology and Immunohistochemistry

Three-micrometer-thick heart sections were deparaffinized and rehydrated by passage through xylene and graded ethanol and then washed twice with 0.05 mol/l Tris-HCl, 0.15 mol/l NaCl, and 0.03% Tween 20 (TBT). Endogenous peroxidase was quenched by incubation of sections in 0.3% H2O2 in methanol for 15 min. Nonspecific binding was blocked by incubation in 10% normal goat serum (NGS) or 5% dry milk in TBT. Fibrillin-1 and fibronectin were detected by overnight incubation with polyclonal antiserum produced in rabbits against a recombinant COOH-terminal half (rf6H) of human fibrillin-1 (58) and a mouse anti-fibronectin antibody (MAB 1926, Chemicon International, Mississauga, Ontario, Canada) diluted 1/200 in TBT containing 10% NGS in a humidified chamber. Anti-α5-integrin antibody from our laboratory (7) and smooth muscle α-actin (clone 1A4, Sigma-Aldrich) were diluted at 1/400 and 1/1,000, respectively. The primary antibodies were revealed by either a peroxidase-bound secondary antibody or biotin-avidin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with 1 mg/ml diaminobenzidine tetrahydrochloride and 0.1% H2O2 as substrates. Sections were counterstained with hematoxylin (Vector Laboratories) and subsequently dehydrated in graded ethanol solutions, transferred to xylene, and mounted in Permount. Slides were visualized with a Zeiss Axioshot 100M microscope (Carl Zeiss Microimaging, Thornwood, NY), and digital images were analyzed with Northern Eclipse image analysis software (Empix Imaging, Mississauga, ON). Nonspecific staining was verified by the absence of the primary antibody.

Fibillar collagen in tissue sections was detected by Picrosirius red staining (25). Interstitial fibrillin-1 and collagen volume fractions were measured by analyzing staining intensity per pixel in multiple fields (15–20 fields/section, 2 sections/animal, and 6–10 rats or 3 mice/group). Elastin fibers were detected after tissue slices were processed with orcein-picroindigocarmin, which stains nuclei brown, elastic fibers rust-brown, collagen blue, and myocytes yellow-green (53).

Real-Time RT-PCR Fibrillin-1 mRNA Analysis

Real-time RT-PCR was performed in a two-step manner. cDNA synthesis and real-time detection were carried out in a PerkinElmer Gene Amp PCR system and with a Mx4000 system (Strategene, La Jolla, CA), respectively. Total RNA was extracted from cardiac tissue according to the TRIzol reagent protocol (Invitrogen, Carlsbad, CA). mRNA (5 μg) was reverse transcribed in a final volume of 20 μl containing 1 μl of 10 mmol/l dNTP, 4 μl BRL 5X buffer, 0.5 μl oligo-(d)T12–18 primer (0.5 μg/μl, Invitrogen), 2 μl of 0.1 mol/l DTT, and 1 μl of 200 U/μl Maloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen) for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. The QuantiTect SYBR Green PCR kit (Qiagen, Mississauga, ON) was utilized in subsequent PCR assays according to the manufacturer’s protocol. Gene expression was compared by the determination of target quantities in experimental samples relative to a calibrator sample (control), and expression levels were corrected for a normalizer gene, 16S rRNA. Sequence-specific primers (BioCorp, Montreal, QC) were designed with Primer Express Version 2 software (Applied Biosystems, Foster City, CA). Single-strand cDNA (2 μl) was used for real-time PCR to amplify a 174-bp fragment of fibrillin-1 cDNA with the complementary antisense primer 5‘-TGTGTCCAAGCGGGGACTTTG-3’ and the sense primer 5‘-CCCTGCGAGATGTGTCCTGC-3’. For a 107-bp fragment of the housekeeping gene ribosomal protein S16 cDNA, the antisense primer was 5‘-GTACCCAGGCTTGAATG-3’ and the sense primer was 5‘-AGGACCGTTTCTGTTGG-3’. To validate our real-time PCR protocol, gene-specific standard curves for fibrillin-1 and S16 were generated from serial 10-time dilutions of cDNA. Linearity for both fibrillin-1 and S16 ranged from 1/10 to 1/10,000. Slopes were similar (−3.05 for fibrillin-1 vs. −3.08 for S16), indicating a threefold increase in the amplification product at every cycle. Thus 2 μl of 1/100 cDNA mixture were amplified with specific primers at a final concentration of 1 mmol/l. All real-time RT-PCRs were performed in duplicate and were conducted with an initial denaturing interval (95°C, 15 min) and then with 40 sequence cycles for fibrillin-1 and S16: 94°C (30 s), 55°C (45 s), and 72°C (30 s). Amplification products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized with UV light to verify fragment length and the absence of nonspecific products that could interfere with the fluorescence signal produced by SYBR green.
Melting-curve analysis was also performed to control for the absence of primer dimers.

**Fibrillin-1 Expression by Cultured Cardiac Fibroblasts**

Fibroblasts from the heart ventricles of 200- to 250-g male Sprague-Dawley rats were prepared as described previously (14). They were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.1% BSA and 10% fetal bovine serum until they reached 60–80% confluence (4 days). The subconfluent cells were then fasted in DMEM and 0.1% BSA in the absence of serum for 24 h before stimulation. Quiescent cells served as controls. Cells were used between passages 1 and 4.

When secreted, ECM proteins (fibrillin-1, collagen type 1, and fibronectin) assemble to form insoluble microfibrils that deposit on the surface of cultured dishes. Deposited ECM proteins were thus measured by an in-cell-based assay. Freshly synthesized soluble ECM proteins were detected by SDS-PAGE after the addition of SDS sample buffer, followed by immunoblotting (56). When assayed by Western blot analysis, quiescent cells in 24-well plates were stimulated for 48 h in the presence of $10^{-10}$ to $10^{-8}$ mol/l ANG II or $10^{-13}$ to $10^{-10}$ mol/l transforming growth factor (TGF-$eta$1). After the medium was removed and washed with PBS, the cells were harvested in SDS sample buffer. For in-cell-based assay, the cells were seeded in 96-well plates and after 24 h in serum-free medium they were stimulated with ANG II or TGF-$eta$1. After 48 h, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. They were then incubated with the primary antibody [rabbit anti-fibrillin-1 or rabbit-anti-collagen type-1 (AB755p, Chemicon International) or rabbit anti-fibronectin and mouse anti-$eta$-actin (clone AC-15, Sigma-Aldrich)]. In both Western blot analysis or in cell-based assay, the bands or wells were quantified in an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) by using goat anti-rabbit Alexa Fluor 680 (Molecular Probes, Eugene, OR) and goat anti-mouse IRDye800 (Rockland Immunochemicals, Gilbertsville, PA). Immunoblot data were normalized with an anti-mouse GAPDH antibody (ab8245, Abcam Limited, Cambridge UK), and in-cell data, with anti-$eta$-actin antibody.

**Interaction Between Integrins and Fibrillin-1**

The possible interaction between fibrillin-1 and RGD-dependent integrins on cardiac fibroblasts was examined by a radioligand displacement assay previously described (57). In brief, Nonidet-P40-solubilized, cultured cardiac fibroblasts were incubated with $^{125}$I-labeled echistatin in the presence of increasing concentrations of fibrillin-1 polypeptides. These polypeptides were produced by recombinant expression in mammalian cells as described (24). The structure of the fragments is presented in Fig. 7. The SDS-stable echistatin-integrin complexes were then separated by SDS-PAGE, and radioactivity was quantified by the Storm 860 system (Molecular Dynamics, Sunnyvale, CA). Cardiac fibroblasts present three different radioactive bands that correspond to $\alpha\beta_1$, a mixture of $\alpha\beta_1^-$ and $\alpha\beta_2^-$, and $\alpha\beta_1^-$ integrins. The interaction between the fibrillin-1 fragments and each radioactive band was evaluated by displacement curves. The potency of each peptide was measured according to the concentration resulting in 50% displacement.

**Statistical Analysis**

The data were expressed as means ± SE. All values were subjected to Student’s $t$-test or to one-way ANOVA, followed by the Bonferroni $t$-test. $P < 0.05$ defined statistical significance.

**RESULTS**

**Fibrillin-1 Distribution in Normal Rat Hearts**

The distribution of fibrillin-1 in the rat myocardium was evaluated by immunohistochemistry. As seen in serial sections illustrated in Fig. 1, each ECM component, collagen (Fig. 1A), fibrillin-1 (Fig. 1B), and fibronectin (Fig. 1C) had a relatively similar distribution throughout the myocardium. The spatial arrangement of fibrillin-1 forms a highly structured network and resembles collagen more closely than fibronectin distribution. In normal hearts, fibrillin-1 appeared mainly as large strands of waves around a group of myocytes (Fig. 1B, open arrow) and vessels (Fig. 1B, solid arrow), while fibronectin formed direct connections between adjacent cardiomyocytes and surrounded the coronary artery endothelium and ECM of capillary, arterial, and venous endothelial cells. Although fibrillin-1 microfibrils are a normal constituent of arteries (46), in the present study we were unable to detect them in the media because the fibrillin-1 epitopes are probably masked. Protease

![Fig. 1.](http://example.org/fig1.jpg) Distribution of fibrillin-1 in normal rat myocardium. A: collagen staining (picrosirius staining); B: fibrillin-1 (immunostaining) (solid arrow showing perivascular area and open arrow perimysium); C: fibronectin (immunostaining); D: elastin staining (orcein-picroindigocarmin staining). Microphotographs are representative of results obtained from 3 to 5 sections per rat with $n = 6–10$ rats per group. Scale bar: 100 $\mu$m for all images.
digestion failed to reveal them (D. Reinhardt, personal communication). Interstitial elastin staining was faint in the myocardium and was mostly localized around the endothelium in the vessel walls (Fig. 1D, solid arrow). In cardiac valve leaflets, known as a rich source of elastin and myofibroblasts (49, 54), a strong fibrillin-1 immunostaining was also detected (result not shown).

**Fibrillin-1 Expression in Experimental Fibrosis**

After a 14-day ANG II infusion, immunohistochemistry with anti-fibrillin-1 antibody showed, compared with controls (Fig. 2, A and B), dramatic increases of fibrillin-1 expression in the rat myocardium (Fig. 2, C and D). There was also a strong increase of perivascular labeling after treatment. Staining intensity represented a volume fraction of 13.8 ± 1.1% compared with 4.92 ± 0.35% for the controls (Fig. 3).

In the myocardium of DOCA-salt rats, an enhanced pattern of fibrillin-1 distribution was also observed (Fig. 2, E and F). Staining was markedly increased in the interstitium (11.9 ± 0.9% vs. 2.70 ± 0.57% in control rats) (Fig. 3). Fibrillin-1-positive fibers coursed in the long axis of the myocytes to which they appeared to be attached (Fig. 2F, solid arrow).

![Fig. 2. Enhanced fibrillin-1 immunoreactivity after 14-day ANG II infusion or DOCA-salt treatment. Compared with control rats (A and B), ANG II-infused rats showed more intense fibrillin-1 labeling in the interstitium (C) and around the vessels (D). Fibrillin-1 immunoreactivity of DOCA-salt rats was intense in the interstitium (E) and around the arteries (F) and extended between the myocytes (double-head solid arrow showing perimysium). Loci of reparative fibrosis were identified as fibrillin-1 positive (G, solid arrow). Elastin deposition (H, solid arrow showing rust brown) was noted in the center of the scar in DOCA-salt myocardium. Microphotographs are representative of results obtained from 3 to 5 sections per rat with n = 6–10 rats per group. Scale bars: 100 μm for all images.](http://ajpheart.physiology.org/)

![Fig. 3. Quantification of interstitial fibrillin-1 and collagen. Fibrillin-1 and collagen deposition in the myocardium of ANG II-infused rats, DOCA-salt rats, and transgenic mice were derived from quantification of the intensity of staining per pixel in each field and represent the mean of 15–20 fields per section, 2 sections per animal, n = 6–10 (rats) and 3 (mice) per group, respectively. *P < 0.05 vs. control.](http://ajpheart.physiology.org/)

![Fig. 4. Enhanced fibrillin-1 immunoreactivity in transgenic mice. Compared with control (A and B), an increased perivascular and interstitial fibrillin-1 deposition was detected in 1B (C and D) and 2C (E and F) transgenic mice. Microphotographs are representative of results obtained from 3 to 5 sections per mouse with n = 3 mice per group. Scale bars: 100 μm for all images.](http://ajpheart.physiology.org/)
Immunohistochemical evidence of fibrillin-1 accumulation at sites of microscopic scarring and perivascular fibrosis of coronary arteries appeared in both the right and left ventricles. Fibrillin-1 staining was particularly intense in reparative fibrotic areas (Fig. 2G, solid arrow). Elastin accumulation was often observed in the center of scars in the DOCA-salt myocardium (Fig. 2H, solid arrow) and in the ANG II-infused rat heart (not shown). Fibrillin-1 was distributed evenly throughout the scars without apparent association with elastin.

In 1B normotensive transgenic mice, compared with controls (Fig. 4, A and B), we clearly observed an increased perivascular and interstitial fibrillin-1 deposition (1.09 ± 0.28% to 4.11 ± 0.69%; P < 0.05) (Fig. 3 and Fig. 4, C and D). In 2C hypertensive ANG II-producing mice (Fig. 4, E and F), we detected a sixfold increase of fibrillin-1 immunoreactivity from 1.09 ± 0.28% in control mice to 7.77 ± 0.83%.

These two lines showed no evidence of cardiac necrosis despite dramatically increased cardiac ANG II content (60). Moreover,
these results raise the possibility that ANG II can stimulate fibrillin-1 synthesis in the absence of increase in BP as detected in 1B normotensive mice.

As expected from our immunohistochemistry results, real-time RT-PCR analysis (Fig. 5) confirmed a statistically significant upregulation of fibrillin-1 gene expression in the hearts of ANG II-infused and in DOCA-salt-treated rats (2.5- and 6.6-fold increase, respectively). In 2C transgenic mice, a 2.8-fold increase was observed. However, in 1B transgenic mice, fibrillin-1 gene expression levels were not changed statistically, suggesting that other mechanisms, such as decreased proteolytic degradation, should also be considered in matrix homeostasis.

**Fibrillin-1 Expression by Cultured Cardiac Fibroblasts**

To establish whether ANG II and TGF-β1, both profibrogenic factors, directly stimulate fibrillin-1 synthesis, cultured rat cardiac fibroblasts were treated with increasing doses of ANG II or TGF-β1. Nontreated cells showed a low level of fibrillin-1 expression. Stimulation with ANG II or TGF-β1 augmented soluble fibrillin-1 (Fig. 6A) and deposition (Fig. 6B) in a concentration-dependent manner. Fibrillin-1 deposition was also accompanied by collagen type I and fibronectin. In cultured cardiac fibroblasts, ANG II and TGF-β1 stimulated fibrillin-1 mRNA expression by 2.04 ± 0.22- and 1.62 ± 0.13-fold over basal levels, respectively (Fig. 6C).

**Interactions Between Fibrillin-1 and Cardiac Fibroblasts**

Further examination by immunohistochemistry at higher magnification of myofibroblasts, as detected by smooth muscle α-actin and α5β1-integrin (Fig. 7, A, B, and E), revealed that these cells are in close contact with fibrillin-1 microfibrils (Fig. 7, C and F), suggesting that this macromolecule can indeed interact with cardiac fibroblasts (and myofibroblasts) through cell surface receptors. To verify the interaction of fibrillin-1 with RGD-dependent integrins of cardiac fibroblasts, an established competition assay tested which fibrillin-1 polypeptides could displace radiolabeled echistatin, a RGD disintegrin ligand (55). Fibronectin, a ligand known to interact with all RGD-dependent integrins, served as an internal standard. Among the different fibrillin-1 fragments that we tested, only fragment rF6H possessed an RGD motif (Fig. 8A). A typical displacement radioautogram with polypeptide rF51 is shown in Fig. 8B. Our results on α5β1-integrin (Fig. 8C) indicate that F6H can displace 125I-labeled echistatin but at a lower potency than fibronectin, suggesting that the RGD motif may possibly be involved in the interaction of fibrillin-1 with RGD-dependent integrins. Interestingly, fragment rF16 and a shorter internal fragment rF51 could also displace the radiolabeled disintegrin with potency lower than that of fibronectin. Displacement of 125I-labeled echistatin by all the fragments occurred in a

**Fig. 7.** Fibrillin-1 and myofibroblasts. Serial sections, immunostained for smooth muscle α-actin (A and B) and fibrillin-1 (C) and stained with picro-sirius red for collagen (D), revealed that myofibroblasts are at proximity of fibrillin-1- and collagen-positive fibrotic regions of DOCA-salt-treated rats. Close localization of α5-integrin-positive fibroblasts (E, black arrows) and fibrillin-1 microfibrils (F) was detected in serial sections. Microphotographs are representative of results obtained from three sections per rat with n = 6–10 rats per group. Scale bars: 100 μm for A, B, and D, 25 μm for C, and 50 μm for E and F.
In many elastic tissues, such as arteries, fibrillin-1, being the major constituent of microfibrils, is observed in close association with amorphous elastin. Except in valvular leaflets and in coronary arteries, the proportion of elastin in the interstitial myocardium is considerably less (16), and elastin does not appear to be an important constituent on this interstitial matrix.

This study reports a significant increase in fibrillin-1 synthesis in three experimental models of cardiac fibrosis. The use of these models allows discrimination between local tissue factors ANG II or ET-1 versus elevated arterial pressure that may affect expression of ECM proteins, including fibrillin-1. In the first model, chronic ANG II infusion resulted in elevated BP, cardiac hypertrophy, and fibrosis (8, 36, 63). In the second model, long-term treatment with DOCA and 1% NaCl, in which plasma renin activity and circulating ANG II are suppressed, elevated BP caused reactive and reparative fibrosis, presumably through an ET-1-dependent process (2, 31). The last model was a transgenic mouse model that expresses a fusion protein resulting in local cardiac ANG II secretion either at levels 30-fold and 1,000-fold higher than normal cardiac ANG II (60). Cardiac hypertrophy was only observed in the highest level-secreting transgenic mouse, whereas fibrosis being detected in both. This model can thus discriminate between the local and systemic effects of ANG II on the heart, more specifically on fibrillin-1 accumulation.

During experimental cardiac fibrosis, fibrillin-1 was overexpressed throughout the myocardium, particularly in fibrotic areas. After ANG II infusion and DOCA-salt treatment, myocardial fibrillin-1 was enhanced and morphologically changed. Because of a higher expression of fibrillin-1, the fibers look thicker, especially those surrounding individual myocytes and groups of myocytes, myofibrils, and muscle fibers. Significant areas of reparative fibrosis occurred after ANG II infusion and in DOCA-salt rats. These sites showed consistently abundant fibrillin-1 deposits. Fibrillin-1 is thus an integral component of the myocardial ECM, and its increased expression is a characteristic of fibrous tissue in the myocardium showing that fibrillin-positive fibers play an important role in the process of tissue repair. These observations correlate with a unique study on spatial arrangements of microfibrils in myocardial scars of human cardiac specimens (61).

Furthermore, fibrillin-1 accumulation varies with the severity and form of structural repair, reactive fibrosis in transgenic mice versus reparative fibrosis seen in the two rat models. The parallel increase of collagen and fibrillin-1 during fibrosis suggests that fibrillin-1 gene modulation of expression is regulated at the same level as the collagen type I gene.

Fibrillin-1 microfibrils demonstrate extensibility properties (28). In the low-pressure circulation, fibrillin-1 microfibrils, in the absence of elastin, confer elasticity to blood vessels (15). The presence of fibrillin-1 in the cardiac interstitium may thus provide elasticity to this tissue. Fibers of collagen type I, the major ECM protein, exhibit high tensile strength and determine stiffness of the working myocardium (23). Extensive accumulation of collagen type I in fibrosis considerably increases rigidity of the ventricular walls and, therefore, limits the effectiveness of contraction and relaxation. Fibrillin-1 overexpression may thus represent a compensatory mechanism that counterbalances collagen fiber stiffness. Measuring elasticity and extensibility of myocardium from Marfan’s syndrome patients or from fibrillin-1 knockout mice would be a way to

**DISCUSSION**

Our immunohistochemistry data revealed that fibrillin-1 is a major connective tissue protein in the normal adult rodent myocardium. It was relatively abundant and expressed throughout the myocardium as thin fibers that crossed over myofibril bundles and around intramural coronary arteries and arterioles. Fibrillin-1-positive fibers were oriented in the longitudinal axis of myocytes, and this spatial arrangement suggested that these microfibrils have an important role in transmitting forces from myocytes to the extracellular connective tissue framework.
define contribution of fibrillin-1 to tissue stiffness. Unfortunately, no such studies have been performed yet.

It is not clear whether these fiber systems are interconnected in the myocardium. However, hitherto no direct interactions between collagen fibers and microfibrils or fibrillin-1 have been reported. Proteoglycans, such as decorin, biglycan, and versican, can interact with both collagen and fibrillin-1 and may thus serve as bridging structures (19, 50). These proteoglycans are indeed expressed in the myocardium (1, 21, 37).

After 2 wk of ANG II infusion (7) and 3 wk of DOCA-salt treatment, marked increases of collagen and fibrillin-1 deposition as well as myofibroblasts were colocalized anatomically in serial sections taken from the same heart. This indicates that myofibroblasts were involved in local fibrillin-1 regulation in the rat myocardium, and their contribution to tissue repair in the injured heart is of considerable interest. Our in vitro results demonstrated that cardiac fibroblasts can synthesize fibrillin-1 mRNA and that ANG II and TGF-β1 enhanced fibrillin-1 secretion and deposition in a concentration-dependent manner. Several reports indicate that ET-1 increases type I and III collagen in cardiac fibroblasts in culture (17, 18, 42). Whether or not ET-1, which is highly expressed in the DOCA-salt model, can stimulate fibrillin-1 synthesis and secretion needs to be tested. ANG II and TGF-β1 are well known to differentiate cardiac fibroblast into myofibroblast by upregulating synthesis of several contractile proteins such as smooth muscle α-actin and tropomysin, and the adhesion receptor α6β1-integrin (51, 56). In addition, ANG II, ET-1, and TGF-β1 have been well documented as key cytokines that promote the accumulation of collagen and other major ECM components in several fibrotic disorders, including cardiac and pulmonary fibrosis, liver cirrhosis, glomerulonephritis, and vascular restenosis (5, 29, 32, 38). Interestingly, TGF-β1-induced ECM deposition in human cardiac fibroblasts occurs largely by increasing ECM gene expression (26). In the reticular dermis of systemic sclerotic skin, TGF-β1 induces fibroblast fibrillin-1 matrix formation (30). TGF-β1 is secreted from cells as a high molecular mass protein complex composed of three proteins, the mature TGF-β dimer, the TGF-β propeptide dimer, or latency-associated protein (LAP), and latent TGF-β-binding protein (LTBP) (45). LTBP is associated with fibrillin-1-containing microfibrils in various tissues including the heart (39). Furthermore, binding studies have detected direct molecular interactions between LTBP-1 and fibrillin-1 (22). All these findings support a role for fibrillin-1 in fibrotic processes.

The hypertensive experimental models are also characterized by local production of reactive oxygen species that can affect ECM protein metabolism (52, 59). Whether or not these reactive oxygen species can play a role in fibrillin-1 synthesis by cardiac fibroblasts remains to be verified.

Our results on 125I-labeled echistatin displacement by fibrillin-1 polypeptides disclosed that fibrillin-1 could indeed interact with all RGD-dependent integrins of cardiac fibroblasts. We have established previously that several RGD-dependent integrins are present on cardiac fibroblasts, including α1β1, α5β1, α6β1, and α5β3 (55). Among them, α5β3-integrin is the most abundant, and its expression is augmented in the myocardium of ANG II-infused rats (7). The results on RGD-bearing fragment rF6H interactions with integrins were expected because the RGD motif has been reported to play a key role as a recognition sequence for α5β1- and α6β1-integrins (3, 10, 41, 47). Although α5β1-integrin can bind several RGD ligands like fibronectin, tenascin vitronectin, osteopontin, and LAP (11, 34, 48), interaction of fibrillin-1 with α5β1 has not been previously reported. An intriguing finding is that one or more of the RGD-dependent integrins found on cardiac fibroblasts can also interact with other portions of fibrillin-1 (fragments F16 and F51) that do not contain any RGD motif. Previous data by others also suggested that the interaction between fibrillin-1 and integrins may not be entirely due to the RGD motif and could involve other sites in fibrillin-1 (3, 47). However, the exact nature of these sites remains elusive.

In summary, the data show that fibrillin-1 contributes to the architecture of the heart. Moreover, its important distribution in the normal heart and its modulation in vivo by ANG II and ET-1 in a pressure-dependent or -independent fashion as well as in vitro by fibrogenic factors suggest that fibrillin-1 is actively implicated in fibrosis and tissue repair, and its increased deposition may affect myocardial stiffness.

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