Myocardial Fibrosis in Chronic Aortic Regurgitation
Molecular and Cellular Responses to Volume Overload

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Background—Myocardial fibrosis is common in patients with chronic aortic regurgitation (AR). Experimentally, fibrosis with disproportionate noncollagen extracellular matrix (ECM) elements precedes and contributes to heart failure in AR.

Method and Results—We assessed [^{3}H]-glucosamine and [^{3}H]-proline incorporation in ECM, variations in cardiac fibroblast (CF) gene expression, and synthesis of specific ECM proteins in CF cultured from rabbits with surgically induced chronic AR versus controls. To determine whether these variations are primary responses to AR, normal CF were exposed to mechanical strain that mimicked that of AR. Compared with normal CF, AR CF incorporated more glucosamine (1.8:1, $P=0.001$) into ECM, showed fibronectin gene upregulation (2.0:1, $P=0.02$), and synthesized more fibronectin (2:1 by Western blot, $P<0.06$; 1.5:1 by affinity chromatography, $P=0.02$). Proline incorporation was unchanged by AR (1.1:1, NS); collagen synthesis was unaffected (type I, 0.9:1; type III, 1.0:1, NS). Normal CF exposed to cyclical mechanical strain during culture showed parallel results: glucosamine incorporation increased with strain (2.1:1, $P<0.001$), proline incorporation was unaffected (1.1:1, NS), fibronectin gene expression (1.6:1, $P=0.07$) and fibronectin synthesis (Western analysis, 1.3:1, $P<0.01$; chromatography, 1.9:1, NS) were upregulated.

Conclusions—In AR, CF produce abnormal proportions of noncollagen ECM, specifically fibronectin, with relatively little change in collagen synthesis. At least in part, this is a primary response to strain imposed on CF by AR. Further study must relate these findings to the pathogenesis of heart failure in AR. **(*Circulation*. 2002;105:1837-1842.)**

Key Words: valves ■ heart failure ■ regurgitation ■ molecular biology ■ myocardium

Myocardial fibrosis is common in patients with aortic regurgitation (AR) who undergo aortic valve replacement.[1,2] Although sequential data in humans are unavailable, observational studies with biopsy material from cardiac catheterization and surgery suggest that fibrosis precedes and may be related to the development of congestive heart failure (CHF) in AR.[1–3] With an animal model system that closely mimicked the pathophysiology of chronic AR in humans, we recently reported data consistent with these clinical observations, finding that fibrosis precedes CHF and is particularly marked when CHF has developed.[4] The myocardium in these experimental animals revealed normal collagen content[5] despite histologically severe fibrosis, suggesting disproportionate accumulation of noncollagen elements within the fibrotic myocardium. Subsequent exploratory analysis with differential display polymerase chain reaction in cardiac fibroblasts (CFs) from animals with chronic AR indicated upregulated expression of several genes that code for noncollagen extracellular matrix (ECM) proteins.[6] Elucidation of the ECM response to AR is potentially important; myocardial fibrosis may be involved in the pathogenesis of CHF[7,8] or may modulate the disordered hemodynamics imposed by myocyte dysfunction.[9] In either case, knowledge of the cellular and molecular bases of fibrosis in AR can enable measures to beneficially modify this process and to recognize imminent left ventricular (LV) dysfunction with increasing precision.[9] Therefore, we used cultured CFs from our model system to define cellular and molecular ECM variations associated with chronic AR. To test the hypothesis that these variations are, at least in part, primary responses to volume overload, assessments were repeated in isolated normal CFs exposed in culture to mechanical stresses modeling those found in vivo in AR.

Methods

Protocol

New Zealand White (NZW) rabbit (Hazelton, Princeton, NJ) models of chronic AR were surgically created and paired with normal controls for subsequent analysis (Figure 1). After euthanization, LV fibroblasts were isolated and cultured. AR and normal fibroblasts were compared for substrate incorporation into ECM, gene expression, and synthesis of specific ECM proteins. Comparisons were repeated in normal LV fibroblasts cultured with or without cyclic mechanical strain.

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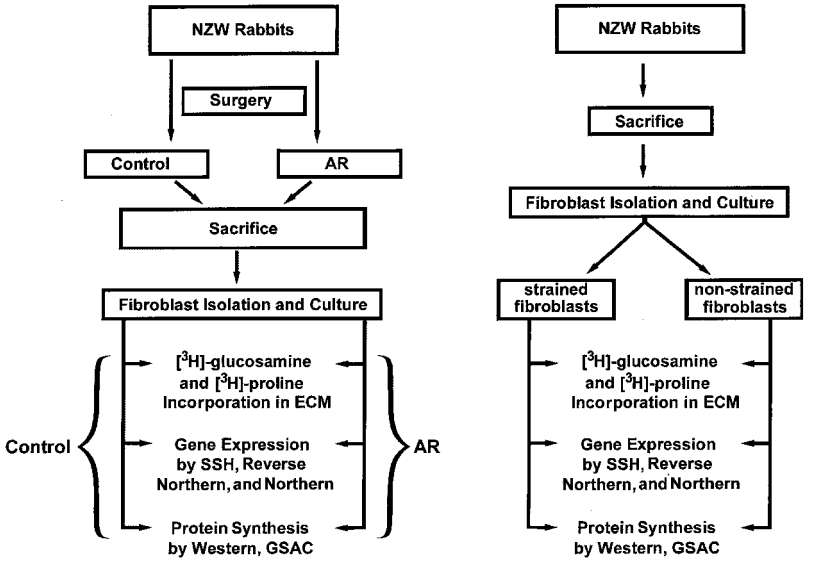

Figure 1. Experimental protocol for comparisons of CFs. Left, Fibroblasts from AR hearts vs controls without AR. Right, Normal CFs cultured with vs without exogenous mechanical strain.

Animals

In 6 rabbits, our previously reported closed-chest Doppler-guided method[10] was used to create AR (mild=regurgitant fraction [RF] <25%, n=1; moderate=RF 25% to 50%, n=3; severe=RF >50%, n=2). Six normal age and weight-matched NZW rabbits were used as controls; 2 rabbits were prepared by sham operation[10] and 4 rabbits were not instrumented. Subsequently, all rabbits followed identical protocols until they were killed (AR after 36, 91, 95, 109, 114, and 122 weeks; controls after 32, 64, 87, 92, 111, and 227 weeks; average weight at death AR=4.5±0.4 kg, control=4.3±0.5 kg, NS). Also, CFs isolated from 3 normal NZW rabbits (weight at death 4.3±0.1 kg) were cultured during application of mechanical strain and, for control observations, without mechanical strain.

Isolation of Cardiac Fibroblasts

Fibroblasts were isolated by a modification[11] of the procedure of Mitra and Morad.[12] Indirect immunofluorescence microscopy confirmed cell lines as fibroblasts.[13] All experiments used cells from passage 6 to assure absence of contamination by residual nonfibroblast lines and of abnormal morphology seen above passage 8.[11] All cultures were grown to confluence before measurements were made. AR and normal cell lines were paired, processed in parallel, and analyzed identically. (One pair was sectioned for regional assessments [not reported herein] precluding some specific comparisons in Table 1.)

Incorporation of [^{3}H]-Proline and [^{3}H]-Glucosamine

Either [^{3}H]-proline, a primary component of collagen, or [^{3}H]-glucosamine, a major component of proteoglycans and glycosaminoglycans, was added to fibroblast cultures. Incorporation in ECM was determined after 24-hour exposure, normalized for cell protein, and adjusted for background activity, as previously described.[14]

Gene Expression

To define targets for assays of abnormally expressed ECM proteins, alterations in gene expression were sought in the same cell lines studied for substrate incorporation with suppression subtractive hybridization (SSH) and reverse Northern and Northern analyses.

Suppression Subtractive Hybridization

Total RNA was isolated from fibroblasts using a modification of the method of Chomczinski and Sacchi;[15] SSH was performed according to the method of Diatchenko et al.[16] Differentially expressed cDNA fragments were cloned directly into T/A cloning vectors, transformed into bacteria, released by restriction enzyme digestion, and used as targets in reverse Northern blots and in probes against Northern blots of normal and AR total RNA.

Reverse Northern Analysis

To confirm differential expression, equal amounts of amplified polymerase chain reaction products were slot-blotted onto nylon membranes, probed with normal or AR ^{32}P-labeled first-strand DNA, and analyzed by autoradiography for hybridization using standard methods.[17]

Northern Analysis

Total RNA isolated from normal and AR CFs was probed with differentially expressed cDNA fragments identified from SSH and reverse Northern analysis.[18] The band intensities of a specific transcript were quantified by computerized scanning of the autoradiogram. Band intensities that corresponded to the GAPDH hybridization of the same Northern blot were similarly determined. (Differentially expressed plasmid clones were sequenced and identified by comparison with GenBank data.)

Matrix Fibronectin and Collagen Synthesis

Although several genes are abnormally expressed (full list to be reported separately), we determined the product only of the upregulated fibronectin gene to prove the principle that ECM synthesis is altered by AR consistent with a substrate incorporation pattern. (Fibronectin gene codes for the most ubiquitous noncollagen ECM protein.) Western analysis and gelatin Sepharose affinity chromatography (GSAC) were used. Although genes that code for collagen were not abnormally expressed, confirmatory Western blot analyses were performed for α1 isoforms of type I and III collagens.

Western Blotting

After cultured fibroblasts reached confluence, serum-free conditioned medium was recovered and analyzed with mouse anti-human fibronectin monoclonal antibody IgG and peroxidase-conjugated rabbit anti-mouse IgG; bands were detected by enhanced chemiluminescence, visualized by exposure to x-ray film, and assessed by densitometry. Band intensities were normalized to corresponding cell protein concentrations. To confirm that collagen synthesis was unchanged, media proteins from 4 cell line pairs (RF=14%, 25%, 45%, and 72%, plus controls) were probed with mouse anti-human collagen type I and type III monoclonal IgG and with peroxidase-conjugated rabbit anti-mouse IgG and analyzed as above.

Gelatin Sepharose Affinity Chromatography

To confirm Western blot analysis results, glucosamine incorporation into fibronectin in the conditioned media from fibroblasts incubated

TABLE 1. Substrate Incorporation and Protein Synthesis in Extracellular Matrix and Gene Expression in Cardiac Fibroblasts From AR vs Normal Hearts

Regurgitant Fraction	[^{3}H]Glucosamine Incorporation, Average cpm/mg Total Protein)	[^{3}H]Proline Incorporation, Average cpm/mg Total Protein	Fibronectin Gene Expression by Northern Analysis, FN:GAPDH	Fibronectin by Western Analysis, Average Densitometry Units*	Fibronectin by GSAC, cpm/mg Total Protein	Collagen I by Western Analysis, Average Densitometry Units*	Collagen III by Western Analysis, Average Densitometry Units*
14%	69 950	838 299	2.07	14 888	5915	20 229	16 774
0	53 210	670 281	0.73	15 297	4705	17 611	26 526
AR:Normal ratio	1.3:1	1.3:1	2.8:1	1.0:1	1.3:1	1.1:1	0.6:1
25%	44 407	908 664	1.07	12 893	4957	23 112	46 498
0	18 233	861 236	0.64	7647	3285	37 663	32 109
AR:Normal ratio	2.4:1	1.1:1	1.7:1	1.7:1	1.5:1	0.6:1	1.4:1
38%	22 454	503 464	3.60	8686	4556	ND	ND
0	14 058	495 448	1.23	2562	3810	ND	ND
AR:Normal ratio	1.6:1	1.0:1	2.9:1	3.4:1	1.2:1	ND	ND
45%	ND	ND	1.20	3995	920	8921	5114
0%	ND	ND	0.80	1 874	770	10 487	5 398
AR:Normal ratio	ND	ND	1.5:1	2.1:1	1.2:1	0.9:1	1.0:1
57%	14 780	ND	3.59	ND	4265	ND	ND
0	8 313	ND	2.49	ND	1628	ND	ND
AR:Normal ratio	1.8:1	ND	1.4:1	ND	2.6:1	ND	ND
72%	ND	ND	1.70	6989	2720	3498	9774
0%	ND	ND	1.20	4424	1990	3043	9641
AR:Normal ratio	ND	ND	1.4:1	1.6:1	1.4:1	1.2:1	1.0:1

FN indicates fibronectin; GADPH, glyceraldehyde 3-P-dehydrogenase; and ND, not determined.

*Equal amounts of total cell protein were loaded in all AR and normal lanes of any single gel; therefore, densitometry units for any AR:normal ratio are normalized to identical total cell protein values.

with [^{3}H]-glucosamine (see above) was determined by GSAC.[19] [^{3}H]-Glucosamine eluted from each sample was normalized to the respective cell protein content. All assays were performed identically in paired AR and normal cultures; relative fibronectin content was determined as the ratio of cpm/mg cell protein (AR:normal).

Strained Fibroblast Cultures

Continuous cycles of stretch (strain)/relaxation were applied to normal CFs in culture as previously described.[20] Cells grown on flexible, collagen-coated membranes were exposed continuously to 60 cycle/min equibiaxial stretching (maximal=25%, modeling diastolic strain of AR; minimal stretch=10%, modeling systolic strain of AR) for 4 days. Strains were those expected at LV midwall with LV dimensions and pressures typically seen in our rabbits with severe, chronic AR, derived from our validated LV wall stress model.[21]

Statistical Analysis

Paired *t* tests (2-tailed) were used to determine differences in glucosamine and proline incorporation, fibronectin gene expression, and protein synthesis among AR fibroblast cultures versus paired normals and stressed versus nonstressed normals. Each replication of an AR or normal analysis was used in statistical calculations because replications were equal for all comparisons. Paired *t* tests and the Wilcoxon signed rank test, as appropriate, were also used to determine the effect of strain on experimental variables. The potential confounding influence of cell line or age at death was evaluated by repeated-measures ANOVA. $P < 0.05$ was considered statistically significant.

Results

At death, age of control animals (124.0±55.3 weeks) was statistically indistinguishable from AR animals (114.0±11.8 weeks, NS). Clinically, no animal had CHF; inspection of rabbit lungs at death revealed modest pulmonary vascular congestion only in the 2 rabbits with severe AR.

Cardiac Fibroblasts: AR Versus Controls

Substrate Incorporation

Compared with their paired normal CF cultures, AR fibroblast cultures invariably incorporated more [^{3}H]-glucosamine in ECM proteins (average ratio 1.8:1, $P=0.001$; Figure 2,

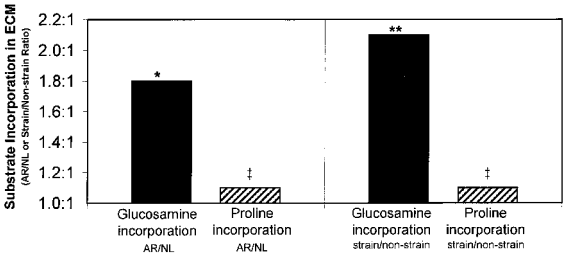

Figure 2. Glucosamine or proline incorporated into ECM by CF. Left, Incorporation by CF from AR hearts, expressed as a ratio normalized to incorporation by normal CF. Right, Incorporation by normal CF cultured during exogenous mechanical strain, expressed as a ratio normalized to incorporation by normal CF cultured without mechanical strain. *$P=0.001$, glucosamine incorporation by AR CF vs paired normal CF. **$P<0.001$, glucosamine incorporation by strained normal CF vs paired normal nonstrained CF. ‡NS, proline incorporation by AR CF vs paired normal CF and strained normal CF vs nonstrained CF.

TABLE 2. Substrate Incorporation and Protein Synthesis in Extracellular Matrix and Gene Expression in Normal Cardiac Fibroblasts Cultured With vs Without Exogenous Mechanical Strain

Cell Line	With (+) or Without (−) Mechanical Strain	[^{3}H]Glucosamine Incorporation, Average cpm/mg Total Protein	[^{3}H]Proline Incorporation, Average cpm/mg Total Protein	Fibronectin Gene Expression by Northern Analysis, Average FN:GAPDH	Fibronectin by Western Analysis, Average Densitometry Units*	Fibronectin by GSAC, cpm/mg Total Protein
Normal 1	+	38 668	248 978	1.6	10 132	5334
Normal 1	−	17 212	262 363	1.3	6 141	2065
	+:− ratio	2.2:1	0.9:1	1.2:1	1.7:1	2.6:1
Normal 2	+	33 726	248 780	2.0	30 304	4836
Normal 2	−	15 912	182 722	1.6	25 369	2776
	+:− ratio	2.1:1	1.4:1	1.3:1	1.2:1	1.7:1
Normal 3	+	18 694	312 023	1.7	24 505	1898
Normal 3	−	9820	350 924	0.9	22 546	1368
	+:− ratio	1.9:1	0.9:1	1.8:1	1.1:1	1.4:1

FN indicates fibronectin; GAPDH, glyceraldehyde 3-P-dehydrogenase.

*Equal amounts of total cell protein were loaded in all AR and normal lanes of any single gel; therefore, densitometry units for any +:− ratio are normalized to identical total cell protein values.

Table 1). In contrast (Table 1, Figure 2), [^{3}H]-proline incorporation in AR cultures was indistinguishable from normal (ratio=1.1:1, NS).

Gene Expression

In total, 6 genes were either upregulated or downregulated in AR CFs compared with normal (reported preliminarily in a smaller study[6]). Three of these, all upregulated, coded for noncollagen ECM elements; genes that coded for collagen isoforms were not abnormally expressed by SSH and, therefore, were not tested by Northern analysis. Fibronectin, one of the 3 upregulated genes, was abnormally expressed in all 6 AR cell lines (Table 1, average ratio [fibronectin/GAPDH$_{AR}$: fibronectin/GAPDH$_{normal}$]=2.2:1, P=0.02).

Fibronectin and Collagen Synthesis

To confirm that AR ECM is disproportionately high in noncollagen proteins (suggested by prior studies[5] and by the substrate incorporation results), further analysis focused on fibronectin and collagen synthesis.

Both Western blot and GSAC analyses indicated greater fibronectin synthesis in AR than in paired normal cell lines (by 2.0:1 [P<0.06] and 1.5:1 [P=0.02], respectively; Table 1). Neither cell line nor age at death interacted significantly with the effects of strain on fibronectin synthesis. In contrast, synthesis of collagen types I and III was statistically indistinguishable in AR versus normal (0.88:1 [type I], 1.04:1 [type III], NS; Table 1).

Normal Cardiac Fibroblasts Strained in Culture

ECM glucosamine incorporation, fibronectin gene expression, and fibronectin synthesis all were greater in strained versus nonstrained fibroblasts (Table 2), which paralleled results in AR versus normal fibroblasts. Thus (Table 2, Figure 2), glucosamine incorporation was upregulated by strain in all 3 cell lines (average 2.1:1, P<0.001), whereas proline incorporation was unaffected by strain (1.1:1, NS). Fibronectin gene expression (1.6:1, P=0.07; Table 2) and fibronectin synthesis (Western blot analysis=1.3:1; P<0.01; GSAC=1.9:1, NS; Table 2) were also upregulated by strain. The relative magnitude of differences between strained versus nonstrained normal fibroblasts was similar to that between AR and control animals for all evaluated parameters (Figure 2, Tables 1 and 2).

Discussion

Our findings indicate that, with or without CHF, AR is characterized by abnormal ECM production, which features a relative abundance of noncollagen ECM, specifically including fibronectin. This is consistent with our earlier report of histologically evident fibrosis in experimental animals with chronic AR.[4,5] In these animals, myocardial collagen content was normal despite exuberant fibrosis in some animals; noncollagen ECM proteins were not assessed.

Our results are the first of which we are aware to define ECM collagen and noncollagen variations in a model of chronic volume loading or of AR. However, our data are consistent with the lack of increase in LV collagen during short-term (≤2 months) LV volume loading from aortocaval fistulae in dogs[22] and rats.[23–25] Also, although AR was not studied, recent reports suggest stress-induced variant expression of genes involved in ECM metabolism.[26]

Our results contrast with those reported in the pressure-loaded LV in which collagen hyperproduction frequently has been identified[27] and noncollagen ECM usually is not assessed. The biological basis for the apparent difference in LV ECM response to volume and pressure loading is not clear. However, the mechanical stresses that impact on the myocardium differ markedly in AR versus pressure-overload states.[7,9,28] Recent findings suggest that stress-responsive elements in genomic promoters are activated by and mediate cellular responses to mechanical stresses.[29] Although transduction pathways are not yet known, different forms of stress may affect stress-responsive elements differently.

Our results in cultured AR fibroblasts, plus earlier findings of histologically evident fibrosis in the absence of CHF[4] and, occasionally, in the absence of apparent myocyte damage, suggest that myocardial fibrosis in AR may be, at least in part, a primary response to volume overload. This is supported by

our finding of similar cellular/molecular abnormalities in cultured AR fibroblasts and in normal fibroblasts subjected for several days (beyond the time of immediate gene expression changes) to dynamic, cyclical strain modeling that of AR. Previously, this issue has not been specifically assessed. However, our findings are consistent with recent reports of gene and signaling protein activation[30] and upregulation of fibronectin gene expression[31] by short applications of static tensile strain to isolated rat CFs.

The design of the present study does not permit confident inferences in regard to the relation of these findings to the pathophysiology of CHF in AR. It is well accepted that myocardial fibrosis is likely to depress LV diastolic performance. However, recent data that support the importance of myocyte-ECM interactions in maintaining systolic function[32] suggest that fibrosis may contribute to systolic dysfunction by altering this interaction. Fibronectin variations may be central to such pathophysiology, because this protein mediates connections of cardiomyocytes and ECM collagen.[33] Finally, static stress can alter fibroblast production of integrins,[30] which can mediate physical interaction of cells and ECM. Alternatively, fibrosis may limit the rate of LV dilatation (and, thus, of wall stress escalation) in AR, potentially protecting the myocyte against dysfunction on the basis of contractile energy requirements of increasing wall stresses. Whether fibrosis is destructive or protective, fundamental understanding of ECM biology may enable beneficial alteration of the fibrotic process, whereas definition of the temporal relation of fibrosis and CHF may enhance clinical prognostication (eg, using radiolabeled ligand imaging[9,34]) to optimize the timing of currently available therapies.

Study Limitations

First, experimental AR was surgically created in a species that does not manifest AR naturally. Second, the primary data were obtained from cell cultures maintained in highly circumscribed and nonphysiological environments. Third, it is unclear why molecular and cellular abnormalities were maintained in AR animals through 6 passages of somatic cell divisions despite the absence of the inciting volume load stresses. The latter concern is mitigated by the similarity of findings in normal fibroblasts subjected to strain in culture. Also, recent studies suggest that environmental factors (perhaps including strain) can cause epigenetic changes in transcriptionally active genes, perhaps accounting for maintenance of genetic variations over generations.[35] Fourth, exogenous strain in culture is similar, but not identical, to the strain plus stress found in AR in vivo. We did not model the transmural stress generated by pressure on the LV endocardium during systole. However, transmural stress is common in both pressure and volume overload; circumferential strain predominates in volume overload,[21] but usually it is modest in clinically relevant pressure overload. Therefore, the stresses we used emphasize the stimuli most unique in AR. Also, they were applied uniformly to model severe AR in all strained cultures; hearts with AR in vivo ranged from mild to severe, perhaps accounting for some quantitative differences when results with AR versus normal and strained versus nonstrained normal fibroblasts were compared. Finally, the quantitative variability inherent in our relatively small number of observations permits only limited inferences in regard to the absolute magnitude of the effect of AR and strain on fibroblast cell biology. Nonetheless, most of our comparisons reached statistical significance, which supported the inference that chance, alone, did not account for results. Limitations notwithstanding, our findings strongly suggest that chronic AR directly causes molecular and cellular alterations in CFs that may affect the capacity of the myocardium and of the patient to compensate for AR. Further study must define the specific pathways by which the physical stresses of AR are transduced to altered molecular and cellular biology and must assess the relation between these findings and myocardial and clinical dysfunction in AR.

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