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 [3H]-glucosamine and [3H]-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 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 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.
Animals
In 6 rabbits, our previously reported closed-chest Doppler-guided method 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 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 of the procedure of Mitra and Morad. Indirect immunofluorescence microscopy confirmed cell lines as fibroblasts. All experiments used cells from passage 6 to assure absence of contamination by residual nonfibroblast lines and of abnormal morphology seen above passage 8. 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 assessment of AR and normal cell lines were paired, processed in parallel, and analyzed identically. (One pair was sectioned for regional assessment.

Incorporation of [3H]-Proline and [3H]-Glucosamine
Either [3H]-proline, a primary component of collagen, or [3H]-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.

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 Chomczynski and Sacchi. SSH was performed according to the method of Diatchenko et al. 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 32P-labeled first-strand DNA, and analyzed by autoradiography for hybridization using standard methods.

Northern Analysis
Total RNA isolated from normal and AR CFs was probed with differentially expressed cDNA fragments identified from SSH and reverse Northern analysis. 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 code 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...
with [3H]-glucosamine (see above) was determined by GSAC.19 [3H]-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 stretch 10%, modeling systolic strain) 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 [3H]-glucosamine in ECM proteins (average ratio 1.8:1, *P*<0.001; Figure 2, left, incorporation by AR CF vs paired normal CF). Proline incorporation (Figure 2, right) was statistically indistinguishable between paired AR and normal CF. **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 1. Substrate Incorporation and Protein Synthesis in Extracellular Matrix and Gene Expression in Cardiac Fibroblasts From AR vs Normal Hearts**

<table>
<thead>
<tr>
<th>Regurgitant Fraction</th>
<th>[3H]Glucosamine Incorporation, Average cpm/mg Total Protein</th>
<th>[3H]Proline Incorporation, Average cpm/mg Total Protein</th>
<th>Fibronectin Gene Expression by Northern Analysis, FN: GAPDH</th>
<th>Fibronectin by Western Analysis, Average Densitometry Units</th>
<th>Collagen I by Western Analysis, Average Densitometry Units</th>
<th>Collagen III by Western Analysis, Average Densitometry Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>14%</td>
<td>699 950</td>
<td>838 299</td>
<td>2.07</td>
<td>14 888</td>
<td>5915</td>
<td>20 229</td>
</tr>
<tr>
<td>0</td>
<td>53 210</td>
<td>670 281</td>
<td>0.73</td>
<td>15 297</td>
<td>4705</td>
<td>17 611</td>
</tr>
<tr>
<td>AR: Normal ratio</td>
<td>1.3:1</td>
<td>1.3:1</td>
<td>2.8:1</td>
<td>1.0:1</td>
<td>1.3:1</td>
<td>1.1:1</td>
</tr>
<tr>
<td>25%</td>
<td>44 407</td>
<td>906 664</td>
<td>1.07</td>
<td>12 893</td>
<td>4957</td>
<td>23 112</td>
</tr>
<tr>
<td>0</td>
<td>18 233</td>
<td>861 236</td>
<td>0.64</td>
<td>7647</td>
<td>3285</td>
<td>37 663</td>
</tr>
<tr>
<td>AR: Normal ratio</td>
<td>2.4:1</td>
<td>1.1:1</td>
<td>1.7:1</td>
<td>1.1:1</td>
<td>1.5:1</td>
<td>0.6:1</td>
</tr>
<tr>
<td>38%</td>
<td>22 454</td>
<td>503 464</td>
<td>3.60</td>
<td>8686</td>
<td>4556</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>14 058</td>
<td>495 448</td>
<td>1.23</td>
<td>2562</td>
<td>3810</td>
<td>ND</td>
</tr>
<tr>
<td>AR: Normal ratio</td>
<td>1.6:1</td>
<td>1.0:1</td>
<td>2.9:1</td>
<td>3.4:1</td>
<td>1.2:1</td>
<td>ND</td>
</tr>
<tr>
<td>45%</td>
<td>ND</td>
<td>ND</td>
<td>1.20</td>
<td>3995</td>
<td>920</td>
<td>8921</td>
</tr>
<tr>
<td>0%</td>
<td>ND</td>
<td>ND</td>
<td>0.80</td>
<td>1 874</td>
<td>770</td>
<td>10 487</td>
</tr>
<tr>
<td>AR: Normal ratio</td>
<td>ND</td>
<td>ND</td>
<td>1.5:1</td>
<td>2.1:1</td>
<td>1.2:1</td>
<td>0.9:1</td>
</tr>
<tr>
<td>57%</td>
<td>14 780</td>
<td>ND</td>
<td>3.59</td>
<td>ND</td>
<td>4265</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>8 313</td>
<td>ND</td>
<td>2.49</td>
<td>ND</td>
<td>1628</td>
<td>ND</td>
</tr>
<tr>
<td>AR: Normal ratio</td>
<td>1.8:1</td>
<td>ND</td>
<td>1.4:1</td>
<td>ND</td>
<td>2.6:1</td>
<td>ND</td>
</tr>
<tr>
<td>72%</td>
<td>ND</td>
<td>ND</td>
<td>1.70</td>
<td>6 989</td>
<td>2720</td>
<td>34 984</td>
</tr>
<tr>
<td>0%</td>
<td>ND</td>
<td>ND</td>
<td>1.20</td>
<td>4 424</td>
<td>1 990</td>
<td>3 043</td>
</tr>
<tr>
<td>AR: Normal ratio</td>
<td>ND</td>
<td>ND</td>
<td>1.4:1</td>
<td>1.6:1</td>
<td>1.4:1</td>
<td>1.2:1</td>
</tr>
</tbody>
</table>

*FN indicates fibronectin; GAPDH, 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.
poration was indistinguishable from normal fibronectin/GAPDH normal]

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).

Table 1. In contrast (Table 1, Figure 2), \([\text{H}]\)-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\(_{\text{AR}}\)-fibronectin/GAPDH\(_{\text{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.
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 and upregulation of fibronectin gene expression 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 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. Finally, static stress can alter fibroblast production of integrins, 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 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. 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 strain generated by pressure on the LV endocardium during systole. However, transmural strain is common in both pressure and volume overload; circumferential strain predominates in volume overload, 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.

Acknowledgments
Dr Borer is supported as the Gladys and Roland Harriman Professor of Cardiovascular Medicine at Weill Medical College. Additional support included grants from the Howard Gilman Foundation, New York, NY; Schiavone Family Foundation, Whitehouse Station, NJ; Irving A. Hansen Foundation, New York, NY; Mary A.H. Rumsey Foundation, New York, NY; Charles and Jean Brunie Foundation, Bronxville, NY; David Margolis Foundation, New York, NY; Daniel and Elaine Sargent Charitable Trust, New York, NY; Messinger Family Foundation, New York, NY; and a gift from Stephen and Suzanne Weiss. We gratefully acknowledge the guidance of J.A. Hannafin, MD, PhD, in conceptualizing these studies.

References


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

Jeffrey S. Borer, Sharada Truter, Edmund M. Herrold, Domenick J. Falcone, Magda Pena, John N. Carter, Themy F. Dumlao, Jennifer A. Lee and Phyllis G. Supino

_Circulation._ 2002;105:1837-1842; originally published online April 1, 2002;
doi: 10.1161/01.CIR.0000014419.71706.85

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/15/1837

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/