Dilated Cardiomyopathy Is Associated With Significant Changes in Collagen Type I/III ratio

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Background—It is controversial whether myocardial fibrosis in end-stage dilated cardiomyopathy (DCM) is associated with altered collagen type I/type III (Col I/Col III) ratio.

Methods and Results—Patients with DCM (ejection fraction [EF] <50%, n=12) and with mild global left ventricular dysfunction (EF >50%, n=18) were examined. Col I, Col III, and transforming growth factors-β1 (TGF-β1) and -β2 (TGF-β2) gene expression in endomyocardial biopsies was evaluated by quantitative competitive reverse transcriptase–polymerase chain reaction (qRT-PCR). Collagen content was quantified after picrosirius red and immunohistological staining and by hydroxyproline assay. In patients with EF <50%, there was a pronounced 2- to 6-fold increase of myocardial Col I mRNA abundance (P<0.01), with a corresponding 1.6-fold increase at the protein level versus that found in patients with EF >50%. The Col III mRNA abundance showed a 2.0-fold increase (P<0.04). There was a relevant shift in the Col I/Col III mRNA ratio for DCM patients (Col I/Col III, 8.2) compared with patients with an EF >50% (Col I/Col III, 6.4). In addition, total collagen content was increased in patients with EF <50% (n=3) (4.3±0.1%) compared with patients with EF >50% (n=8) (2.7±0.9%) (P<0.004). The biochemically determined ratio of hydroxyproline/total protein (n=12) was correlated to the Col I mRNA abundance (P<0.05, r=0.77). TGF-β1 and TGF-β2 showed elevated myocardial mRNA abundances (1- to 7-fold and 4- to 5-fold, respectively) in DCM patients.

Conclusions—Differential increase of Col I and Col III leads to an increased Col I/Col III ratio in DCM myocardium. Because Col I provides substantial tensile strength and stiffness, this may contribute to systolic and in particular diastolic dysfunction in DCM. (Circulation. 1999;99:2750-2756.)

Key Words: cardiomyopathy ■ collagen ■ growth substances ■ remodeling ■ polymerase chain reaction

The myocardial extracellular matrix is composed of a complex network of structural protein, mainly collagen type I (Col I) and collagen type III (Col III), which provide architectural support for the muscle cells and also play an important role in myocardial function.1,2 Several studies of dilated cardiomyopathy (DCM) have demonstrated changes in collagen content at the protein level, characterized primarily by an accumulation of Col I.3–5 However, whether there is also an increase in Col III remains controversial. The ratio of collagen types within the heart is of significance because of their different mechanical properties.6 For example, tissues with predominance of Col I (such as tendon) are characterized by strength and stiffness, whereas tissues containing large amounts of Col III (such as skin) are characterized by greater elasticity. Yoshikane et al documented an increase in the number and thickness of collagen fibers in end-stage DCM by electron microscopic and immunohistological methods. A biochemical analysis by Bishop and colleagues3 showed an increase in the absolute amounts of both Col I and Col III. In contrast, Weber et al7 demonstrated a pronounced increase in predominantly thin collagen fibers and a decrease in thick collagen fibers in explanted hearts from patients with end-stage DCM compared with normal myocardium. The apparent discrepancy of these studies may be explained by differences in methodology; however, other factors, such as collagen cross-linking, may have influenced the results.

Our aim was to examine changes in messenger RNA (mRNA) abundance of collagen subtypes in endomyocardial biopsy samples from patients with and without diagnosis of DCM. Furthermore, we also sought to determine whether there were changes in transforming growth factors-β1 (TGF-β1) and -β2 (TGF-β2), cytokines known to play a crucial role in mediating collagen synthesis.8–10 Furthermore, the small amount of tissue available from endomyocardial biopsy samples required that we establish quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) assays to conduct the analysis of multiple genes.

Methods

Patients

Thirty patients with the presumptive clinical diagnosis of DCM were analyzed in this study. All patients underwent cardiac catheterization.
for further evaluation of impaired global or regional left ventricular function initially revealed by echocardiography and by reduced exercise tolerance with a minimum duration of 6 months. Right and left heart catheterization, including standard hemodynamic measurements, was carried out in all patients in whom coronary, hypertensive, and valvular heart disease as well as restrictive or constrictive heart disease had been excluded before an endomyocardial biopsy was obtained. In addition, none of the analyzed patients had decompensated heart failure. Because of the limited number of available biopsies, a subgroup analysis for total collagen content by biopsies of the right ventricular septum in all patients with a Cordis bioptome. From these small biopsies, we measured (1) Col I and Col III mRNA abundance for TGF-

**Biopsy**

Endomyocardial biopsies were taken from the right ventricular septum in all patients with a Cordis bioptome. From these small biopsy samples, we measured (1) Col I and Col III mRNA abundance, (2) mRNA abundance for TGF-β1, TGF-β2, and GAPDH, (3) mRNA abundance for GAPDH, (4) total collagen content histologically by picrosirius red staining, (5) Col I and Col III expression immunohistochemically on the protein level, (6) hydroxyproline concentration/total protein concentration (both µg/mL) was correlated with the collagen mRNA abundance.

All procedures were performed in accordance with ethical standards and with the Helsinki Declaration of 1975. In addition, all patients gave informed consent for all of the invasive studies performed.

**RNA Extraction**

Total RNA was isolated from all 30 samples by a modification of the lithium chloride method according to Auffray et al.11

**Reverse Transcription**

For the PCR, 300 ng total RNA of each biopsy was transcribed into cDNA by use of avian myeloblastosis virus reverse transcriptase (AMV RT) and random primer in accordance with the supplier’s protocol (Promega).

**qRT-PCR**

The qRT-PCR was based on the coamplification of a specific internal standard with the cDNA to be examined. Because of the identical primer sequences of the internal standard and cDNA, both molecules compete for the primers of the PCR mixture in the phase of primer annealing. A decreasing concentration of internal standard accounts for an increased amplification of examined cDNA (Figure 1).

The internal standard was constructed by use of the PCR mimic-constructing kit (Clontech Laboratories, Inc) with linker primers for Col I, Col III, TGF-β1, TGF-β2, and GAPDH. The standard differed in size from the amplified cDNA to distinguish between both PCR products on agarose gels. The difference in size between standard and endogenous cDNA was in the range of 10% to 20% of base length. The exogenous DNA standard was applied in qRT-PCR in 5 dilutions ranging from 0.1 to 0.16 attomol, calculated by an estimated length of 500 bp while a constant concentration of cDNA was added. The amounts of cDNA were 1.5 ng for Col I, 3 ng for Col III, 2 ng for TGF-β1, 7 ng for TGF-β2, and 0.6 ng for GAPDH.

The qRT-PCR program consisted of a denaturing phase at 94°C for 1 minute, primer annealing at 60°C for Col III, TGF-β1, TGF-β2, and GAPDH and at 72°C for Col I, each for 90 seconds, and an extension temperature of 72°C for 2 minutes. The PCR ran for 35 cycles and was still in the linear phase.

The PCR reacting volume of 25 µL consisted of 0.4 µmol/L of each primer, 0.2 mmol/L dNTPs, 2.0 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl, and 4 U of Taq polymerase (Perkin-Elmer).

**Oligonucleotides Used for qRT-PCR**

Primers were selected on 2 different exons, separated by an intron to differentiate between amplified DNA and amplified reverse-transcribed RNA. The primer sequences are listed in Table 1.

**Densitometric Analyses of qRT-PCR Products**

Each qRT-PCR product was loaded on an ethidium bromide–stained agarose gel (1.5%). For analysis of bands, the gel was photographed under 254-nm UV light with a photo documentary system (Ultra Violet Products Gel Documentation System Image Store 5000) and quantified with the NIH image program (Ultra-Violet Products Limited). All values were measured twice to avoid inaccuracy. The

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**TABLE 1. Primer Sequences Used for qRT-PCR**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>5` Region</th>
<th>3` Region</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col I</td>
<td>GGCGGCCAGGGCTCCGAC</td>
<td>AATTCCTGGTCTGGGGCACCC</td>
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</tr>
<tr>
<td>Col III</td>
<td>TGGTTTGGACGCCTGCCA</td>
<td>AGTCTGAGAGATTGCCGCC</td>
<td>483</td>
</tr>
<tr>
<td>TGF-β₁</td>
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<td>TTGCAAGTGTGTATCAGTCTGTC</td>
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<tr>
<td>TGF-β₂</td>
<td>TCCAAAGATTACATCTCGAC</td>
<td>TCCACATGGTTTTTTTCCTCAGTGG</td>
<td>310</td>
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<tr>
<td>GAPDH</td>
<td>TGAAGGTCGAGAGCTCCGACC</td>
<td>CATTGTGGCCATGAGGTCCACCAC</td>
<td>983</td>
</tr>
</tbody>
</table>

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**Figure 1.** Ethidium bromide-stained 1.5% agarose gel of qRT-PCR for TGF-β₁ (lanes 1 through 5: qRT-PCR products; lane M: molecular weight marker; lane 6: negative control; lane 7: positive control cDNA TGF-β₁; lane 8: positive control for DNA standard TGF-β₁).
abundances of all different mRNA species were related to GAPDH mRNA to increase accuracy.

Histology
Picrosirius red staining was used for the histological assessment of the total collagen content by use of computer-assisted morphometry (Lucia software program). Formalin-fixed tissue was processed for paraffin embedding, sectioned at a thickness of 5 μm, and stained with picrosirius red (Polyscience, Inc). The collagen content of the sections was measured when viewed with circularly polarized light according to previously published methods.13 For each sample, a total of 10 fields were analyzed with a ×20 objective lens. Histological analyses of endomyocardial biopsies for myocardial inflammation were performed in a standard manner and evaluated according to the Dallas classification.13

Immunohistology
Endomyocardial biopsies were directly embedded in OCT (Miles Laboratories, Inc) and frozen at −70°C. With a cryostat, the biopsies were cut into 5-μm thin sections, mounted on a slide, and fixed in acetone for 10 minutes.

Antibodies
Biopsies were incubated with monoclonal mouse anti-human antibodies against Col I (Oncogene Research Products) and Col III (Sigma Chemical Co), with a working solution for Col I of 1:300 and Col III of 1:150. Monoclonal antibodies directed against CD3 T lymphocytes were used according to Kühl et al.14

Immunohistological Staining
Each primary antibody was added to a section of 6 slices and incubated for 45 minutes at room temperature in a humidified chamber. After incubation, unbound antibodies were removed by 2 washing steps with PBS for 5 minutes. Then, peroxidase-conjugated rabbit anti-mouse antibody (Dianova GmbH), diluted at 1:200 in PBS containing 10% FCS, was added and likewise incubated for 45 minutes at room temperature. After another 2 washing steps with PBS, the specifically bound antibodies were detected by a stain reaction with carbazole for 12 minutes in the dark. For quantification of stained areas, the sections were viewed with a light microscope at a magnification of ×400 and digitized. Within the Lucia software program (Nikon), the relation between stained and unstained areas was calculated and the Col I and Col III protein was measured as percentage proportion. For each biopsy and antibody, 6 sections were measured and averaged. The quantification of CD3 T lymphocyte content was performed at a magnification of ×400 according to Kühl et al.14

Hydroxyproline Determination
The endomyocardial biopsies were homogenized in 330 μL of 6 mol/L hydrochloric acid for 15 seconds. Of this homogenate, 30 μL was used for the determination of the total protein content in the analyzed tissue sample. Afterward, 300 μL of the 6 mol/L hydrochloric acid homogenate was heated at 120°C overnight to hydrolyze collagen into its component amino acids. Hydroxyproline concentration was measured in an amino acid analyzer in standard fashion. In addition, the total protein concentration of the analyzed biopsy was measured with the bicinchoninic acid protein quantification kit (Pierce). The concentration of tissue hydroxyproline was expressed as the ratio of hydroxyproline concentration/total protein concentration (both μg/mL) for each biopsy. The hydroxyproline concentration was applied to the total protein content, because with the very small size of endomyocardial biopsies, a weight-adjusted standardization of the hydroxyproline content would be not accurate enough.

Statistics
For statistical analysis, the SPSS statistical software package was used. Statistical analyses were done with the Mann-Whitney U test, and the mRNA values of each group are shown with their respective

| TABLE 2. Clinical and Hemodynamic Parameters of the Enrolled Patients |
|--------------------|-----------------|-----------------|--------|
|                    | EF<50% (n=12)   | EF>50% (n=18)   | P      |
| Age, y             | 47±11           | 40±14           | NS     |
| Sex, F/M           | 3/9             | 7/11            | NS     |
| Medication         |                 |                 |        |
| ACE inhibitors     | 7/12            | 6/18            | NS     |
| Diuretics          | 7/12            | 2/18            | <0.05  |
| Digitalis          | 6/12            | 1/18            | <0.05  |
| β-Blocker          | 3/12            | 3/18            | NS     |
| EF, %              | 39±9            | 64±7            | <0.001 |
| CI, L · min⁻¹ · m⁻²| 3.1±1.0         | 4.3±1.0         | <0.004 |
| BSA, mm Hg         | 11±5            | 8±2             | NS     |
| PCWP, mm Hg        | 9±5             | 6±2             | NS     |
| LVEDD, mm          | 63±8            | 51±7            | <0.003 |

CI indicates cardiac index; BSA, body surface area; PCWP, pulmonary capillary wedge pressure; and LVEDD, left ventricular end-diastolic dimension.

Results
Patients and Hemodynamic Parameters
The patients were assigned to 2 groups on the basis of cardiac function. Twelve patients (3 women, 9 men) 34 to 60 years old (mean, 47±11 years) had global left ventricular dysfunction (EF <50%) and were classified as having DCM. Eighteen patients (7 women, 11 men) 17 to 71 years old (mean, 40±14 years) had only mild or regional left ventricular dysfunction (EF >50%) and were classified as having “latent” DCM. The other hemodynamic and clinical data, including the medications, are listed in Table 2.

Collagen Quantification
Gene Expression Studies
The mRNA abundance of Col I, measured as Col I/GAPDH mRNA ratio (0.45±0.49) in the group of patients with an EF <50%, was increased significantly, by 2.6-fold (P<0.01), versus patients with an EF >50% (0.172±0.23) (Figure 2). The mRNA ratio of Col III/GAPDH increased significantly, by 2.0-fold (P<0.04), from 0.027±0.026 in patients with mild regional dysfunction and EF >50% versus 0.055±0.066 in patients with global dysfunction and EF <50% (Figure 2). These changes resulted in a shift in the ratio between Col I and Col III at the mRNA level for patients with DCM (ratio, 8.2) compared with the gene expression ratio of Col I/Col III in the group with an EF >50% (ratio, 6.4).

The growth factors TGF-β1 and TGF-β2 showed a trend toward an increased mRNA abundance in the group with EF <50% (TGF-β1/GAPDH, 0.048±0.027; TGF-β2/GAPDH, 0.018±0.014) versus the group of patients with EF >50% (TGF-β1/GAPDH, 0.029±0.041; TGF-β2/GAPDH, 0.004±0.014).
Histology and Immunohistology of Collagen

The results of gene expression for Col I were confirmed on the protein level. A trend toward a greater accumulation of the percentage proportion of stained and unstained areas of Col I was detected in patients with DCM (Col I, 20±11%) compared with patients with a normal EF (Col I, 12±8%). However, a trend toward a decrease of Col III was found (patients with EF <50%, 30±13%; patients with EF >50%, 36±11%). The histological analysis of the total collagen content by picrosirius red staining visualized in circularly polarized light also showed a significant increase in fibrosis in patients (n=3) with EF <50% (4.3±0.1%) compared with patients (n=8) with EF >50% (2.7±0.9%) (P<0.0035; see Figure 3). This increase in collagen content appeared to be uniform throughout the biopsy samples.

Hydroxyproline Content

Because of the limited availability of endomyocardial biopsies, these biochemical analyses were performed in a subgroup of 12 patients (EF <50%, n=6; EF >50%, n=6; mean EF, 49±11%). The ratio of hydroxyproline concentration/total protein concentration (both μg/mL) was significantly correlated with the mRNA abundance of Col I (r=0.77; P<0.05) (see Figure 4) but not with Col III.

Histology and Immunohistology of Myocardial Inflammation

Active myocarditis according to the Dallas classification was excluded in all patients. The distribution of CD3 T lymphocytes differed between groups; patients with global dysfunction and EF <50% showed a significant (P<0.05) increase of CD3 T lymphocytes of 1.8±0.7 lymphocytes per visual field, compared with the group of patients with EF >50%, who showed a value of 1.3±0.6 CD3 T lymphocytes per visual field.

Collagen mRNA Abundance

The shift in the Col I/Col III ratio from 6.4:1 to 8.4:1 in the patients with DCM may derive from different molecular mechanisms. Either there could be a stimulation of collagen mRNA expression through the activation of various cytokines or there could be a reduced turnover of Col I and Col III mRNAs. Although we found no significant increase in the mRNA abundance of either TGF-β1 or TGF-β2, 2 cytokines intimately involved with collagen production, this does not rule out the former pathway. It is possible that we missed the peak in TGF-β1 and TGF-β2 expression and detected only its consequences. In addition, there are other possible cytokines that may be equally or even more important.

Our study showed a trend toward increased myocardial TGF-β1 and TGF-β2 mRNA abundance in DCM. This, however, could possibly translate into a significant increase in collagen gene expression, because both the promoters of Col I and Col III contain TGF-β–activatable elements.15–17 Because the Col I promoter also contains several SP-1 binding sites, which are not found in the Col III promoter, the differential gene expression levels of Col I and Col III might rely on their respective promoters.18,19 Stimulation of myocardial TGF-β expression itself may result from a number of factors that are not known at present. Because Hinglais et al demonstrated the colocalization of myocardial fibrosis and predominantly T helper lymphocytes and macrophages in spontaneously hypertensive rats, the local myocardial expression of TGF-β1 and TGF-β2 could have been stimulated by the significantly increased CD3 T lymphocytes cell count as seen in our DCM patients with EF <50%.21,22

Collagen Protein Detection

The increase in collagen mRNA that we found was mirrored by an increase in collagen detected at the protein level by histochemistry (picrosirius red staining) and immunohistochemistry. We were able to document a significant increase...
of fibrosis in patients with DCM because of a trend toward an increase in Col I but not Col III in patients with DCM, which is in line with earlier reports by Marijanowski et al.\textsuperscript{4} This increase in collagen content appeared to be uniform throughout the biopsy samples. The inability to detect an increase of Col III by immunohistochemical techniques as well might be influenced by the availability of Col III epitopes for antibody binding. If the Col III fibers are covered by type I collagen, it may be difficult to accurately measure the amount of Col III in these biopsies. Despite this principal methodological problem of immunohistological staining, the immunohistochemical results are consistent with the mRNA findings and indicate a shift in the Col I/Col III ratio on both the mRNA and protein levels. In addition, the biochemical results of the significant correlation between the ratio of hydroxyproline concentration to total protein concentration and the mRNA abundance of Col I emphasize these findings. Although the increase in collagen detected at the protein level may reflect an increase in synthesis, it is also possible that differences in degradation may play a role. Differences concerning protein levels of Col I and Col III may depend not only on differential expression but also on differential degradation. Two different matrix metalloproteinases (MMPs) are described as being active in degrading collagens. MMP 1 is synthesized by

Figure 3. Histological analysis of the total collagen content by picrosirius red staining visualized in circularly polarized light in patients with EF<50\% (A) and EF>50\% (B).
Therefore, the use of right ventricular endomyocardial biopsies from normal hearts for ethical reasons. However, the mean total collagen content in the group of patients with mild left ventricular dysfunction in this study measured by picrosirius red staining in circularly polarized light is similar to the total collagen content measured with the same technique in normal hearts from the right ventricular septum as well as from the free left ventricular wall. Therefore, we also did not analyze the collagen expression in normal hearts. The reported differences in collagen expression between DCM and the patients with mild left ventricular dysfunction are relevant and may have major implications for systolic and in particular diastolic dysfunction as seen in DCM.

Summary
Our results showed increased mRNA abundances for Col I and Col III in endomyocardial tissues of patients with DCM. In addition, a significant shift of the Col I/Col III ratio was found both at the level of gene expression and at the protein level. Finally, the increased mRNA abundances of TGF-β1 and TGF-β3 in DCM suggest that these growth factors could play a role in the myocardial matrix remodeling seen in DCM. An interesting secondary finding was the possibility to establish a method for the analysis of mRNA abundances of 5 different genes in one single human endomyocardial biopsy with a wet weight of 3 to 5 mg.

Limitations
A limitation of this study is that only endomyocardial biopsies from patients with different degrees of impaired left ventricular function were analyzed, and not explanted hearts with end-stage heart failure. Therefore, the analysis of regional distribution of collagen content (septal versus posterior region, endocardial versus epicardial region, right ventricle versus left ventricle), which would have strengthened our study, was not possible. However, this type of analysis of regional matrix remodeling is possible only in explanted hearts with end-stage heart failure, which was not the target group of patients to be evaluated in our study. The aim of the study was to analyze, in particular, the collagen remodeling in endomyocardial biopsies of patients with mild to severe left ventricular dysfunction and not in terminally failing explanted hearts. In addition, postmortem studies by Baandrup et al demonstrated identical collagen contents in the right ventricular septum and the left ventricle as assessed in histological studies of autopsy samples of normal hearts. Therefore, the use of right ventricular endomyocardial biopsies from the right ventricular septum should also be representative for left ventricular matrix remodeling. Further studies will be necessary to determine whether the collagen changes reported here are specific for DCM or whether similar changes occur in heart failure as a result of other cardiac pathology. Another limitation of this study is that we did not obtain endomyocardial biopsies from normal hearts for ethical reasons. However, the mean total collagen content in the group of patients with mild left ventricular dysfunction in this study measured by picrosirius red staining in circularly polarized light is similar to the total collagen content measured with the same technique in normal hearts from the right ventricular septum as well as from the free left ventricular wall. Therefore, we also did not analyze the collagen expression in normal hearts. The reported differences in collagen expression between DCM and the patients with mild left ventricular dysfunction are relevant and may have major implications for systolic and in particular diastolic dysfunction as seen in DCM.

Potential Significance of Collagen Changes
Col I and Col III are essential components of the myocardium, maintaining its structural and functional integrity. Because of their different physical properties, the altered Col I/Col III ratio may therefore have a major impact on the diastolic and systolic function of the heart. Whereas Col III forms an elastic network storing kinetic energy as an elastic recoil, Col I represents a stiff fibrillar protein providing tensile strength. Therefore, increasing Col I protein levels might impose increasing myocardial stiffness, compromising diastolic and systolic function of the myocardium.

References
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