Molecular Normalization of Dystrophin in the Failing Left and Right Ventricle of Patients Treated With Either Pulsatile or Continuous Flow-Type Ventricular Assist Devices

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OBJECTIVES
We investigated the integrity of dystrophin in left ventricle (LV) and right ventricle (RV) of patients with end-stage heart failure due to ischemic cardiomyopathy (IHD) or dilated cardiomyopathy (DCM), and compared the efficacy of pulsatile or continuous flow assist devices on dystrophin reverse remodeling.

BACKGROUND
Recently we demonstrated that the amino (N)-terminus of dystrophin is preferentially disrupted in failing LV myocardium irrespective of the underlying etiology, and that this defect is reversed by mechanical unloading using left ventricular assist device (LVAD) therapy.

METHODS
Myocardial samples were obtained from seven normal controls, seven failing hearts (either DCM or IHD), and 14 failing-heart patients who underwent placement of either pulsatile (7 patients) or continuous flow (7 patients) LVADs for progressive refractory HF. The expression and integrity of dystrophin in these samples were determined by immunohistochemistry using antibodies against the N-terminal and carboxyl (C)-terminal domains of dystrophin.

RESULTS
Immunohistochemical staining identified disruption of the N-terminal dystrophin in both LVs and RVs of all seven failing-heart patients, whereas the C-terminus was normal. Furthermore, this disruption was reversed in 12 of the 14 patients after LVAD therapy using either pulsatile or continuous devices; the degree of the reverse remodeling was similar in both ventricles, although greater recovery was noted in patients treated with pulsatile flow devices.

CONCLUSIONS
Integrity of the N-terminus of dystrophin is a useful indicator of both LV and RV function. In addition to improving LV hemodynamics, LVAD therapy results in amelioration of the myocardial structure of the right cardiac chamber.

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Dystrophin was originally identified as the gene responsible for Duchenne and Becker muscular dystrophies (DMD/BMD) (1) and later for the X-linked form of dilated cardiomyopathy (XLCM) (2). Typically, mutations in the amino (N)-terminus of dystrophin have been reported in patients with XLCM, whereas patients with DMD/BMD have mutations scattered throughout the gene. Dystrophin links the actin cytoskeletal networks and the sarcolemmal dystrophin-associated protein complex (DAPC), which is associated with the extracellular matrix of the cardiomyocyte. Thus, dystrophin plays a key role in the transduction of physical forces in striated muscle (3). Dystrophin also provides an anchorage to the DAPC, as demonstrated in DMD patients in whom primary dystrophin deficiency results in destabilization of the entire complex (4). In addition, the lack of dystrophin is associated with altered structure and mechanical adherence of costameres to the underlying cytoskeletal actin network (5). Therefore, as expected, mutations in dystrophin, actin, or DAPC sub-complexes result in a wide spectrum of skeletal myopathy and/or cardiomyopathy in humans and animal models (6).

In our previous report, we demonstrated a selective abnormality of the N-terminus of dystrophin in the myocardium of the left ventricle (LV) of patients with end-stage cardiomyopathy, regardless of the underlying etiology (7). The integrity of dystrophin in the right ventricle (RV) was not investigated. In addition, chronic mechanical unloading with a pulsatile-type left ventricular assist device (LVAD) resulted in reverse remodeling of dystrophin in the LV, suggesting that there is an important role for dystrophin in the development of ventricular dysfunction and that dystrophin can be used as a molecular marker for heart failure (HF) (7). Although LVAD therapy results in the amelio-
Abbreviations and Acronyms

- BMD = Becker muscular dystrophy
- DAPC = dystrophin-associated protein complex
- DMD = Duchenne muscular dystrophy
- HF = heart failure
- IHD = ischemic cardiomyopathy
- LV = left ventricle/ventricular
- LVAD = left ventricular assist device
- N = amino
- RV = right ventricle/ventricular
- XLCM = X-linked form of dilated cardiomyopathy

ration of the myocardial functional deficits and molecular recovery of the LV, it is unclear whether unloading the LV can help the recovery of the RV. Further understanding of the effects of prolonged LV unloading on the RV structure will increase our understanding of HF and potentially provide markers for myocardial recovery.

In this report, we evaluate the effect of LV mechanical unloading on the failing RV by investigating the molecular remodeling of the N-terminus of dystrophin. We demonstrate that the use of either a pulsatile- or continuous-flow LVAD improves not only the failing LV but also the RV by inducing normalization of dystrophin expression.

METHODS

Patient evaluation. Myocardial samples (RV and LV) from the explanted hearts of seven patients with either ischemic (IHD) or non-ischemic idiopathic dilated cardiomyopathies (DCM) were obtained after informed consent. Left ventricular and RV size and function were measured using M-mode and two-dimensional echocardiographic images, as previously described (8,9). All patients were listed for cardiac transplantation at the Methodist Hospital/Baylor College of Medicine transplant program and were functional class IV using the New York Heart Association classification (10). Endomyocardial biopsy samples from RV and LV free walls were obtained after informed consent from 14 failing-heart patients undergoing treatment with either pulsatile (7 patients) or continuous flow (7 patients) LVADs. Normal myocardial samples (i.e., controls) were obtained from both ventricles by through-cut biopsy of donor hearts before heart transplantation. The Baylor College of Medicine Institutional Review Board fully approved all studies.

Myocardial samples were either snap-frozen in liquid nitrogen or fixed in 2% paraformaldehyde for 45 min, followed by dehydration through increasing concentrations of alcohols, then cleared through xylene for subsequent embedding in paraffin.

Immunohistochemistry. Sections (5 μm) were cut from frozen myocardial samples and stained using anti-dystrophin antibodies (N-terminus- and C-terminus-specific: Novocastra, Newcastle, U.K.) (9). Each primary antibody (diluted 1:10 in phosphate-buffered saline) was incubated with the sections for 30 min at room temperature. After washing, the slides were incubated for 15 min at room temperature with the secondary antibody (FITC-conjugated anti-mouse: Novocastra), diluted 1:400, and then mounted (9).

Analysis by three blinded observers led to a semi-quantitative analysis of the staining intensity. Normal staining was graded as 4, whereas absence was graded as 0. Intermediate staining intensity was graded as 3 (mildly reduced: 50% to 75% of normal), 2 (moderately reduced: 25% to 50% of normal), or 1 (severely reduced: 1% to 25% of normal).

Fixed myocardial tissue sections were stained with picrosirius red as previously described (11,12). Total collagen content was measured as the sum of all areas stained within the slide, including interstitial, perivascular, and microscopic scars. Myocyte size was determined using a computerized edge detection system, as described previously (13). Statistical analysis. Statistical comparisons between controls and pre- and post-LVAD groups were performed with a Mann-Whitney U test to identify statistical significance for myocyte size and collagen expression. Donor heart age difference was calculated by Wilcoxon rank-sum test. All the data in the text and figures are expressed as mean ± SD.

RESULTS

Clinical characteristics of the study cohort. Table 1 shows the demographics of the patients enrolled in the study and from whom end-stage myocardium was obtained. Patients with DCM were younger (49.2 ± 11.7 years) than the IHD patients (53.6 ± 10.3 years), but the difference was not statistically significant, although the power to detect a difference is very low owing to the small number of patients (p = 0.3824). Fourteen patients underwent LVAD placement for progressive cardiac deterioration, seven with pulsatile flow devices (Novacor, World Heart Inc., Oakland, California) and seven with continuous flow devices (DeBakey-Noon Axial Flow VAD, MicroMed Technology, Inc., Houston, Texas). All LVAD-treated patients as well as five of seven failing-heart patients received maximal intravenous inotropic support. In addition, six LVAD-treated patients had inotropic support even after LVAD implantation (Table 1). The duration of inotropic support for each group was the following: failing = 14.9 ± 12.6 days; continuous = 21.0 ± 9.5 days; pulsatile = 36.4 ± 38.9 days. However, this difference was not statistically significant, perhaps owing to the limited cohort studied (p = 0.25).

The mean duration of the LVAD support was 88.0 ± 57.5 days for the patients treated with pulsatile pump, and 40.14 ± 31.2 days for those treated with a continuous flow pump. Also in this case, because of the small number of patients evaluated, the difference was not statistically significant (p = 0.077). Histopathological
analysis of patients who underwent mechanical support with either a continuous or pulsatile device showed reduced total collagen content in both the RV and LV (data not shown), as described elsewhere (14).

**Dystrophin expression in failing myocardium.** Figure 1A, shows representative immunostains for the N-terminus of dystrophin in LV and RV samples obtained from normal and failing myocardium. Strong staining of the sarcolemma is apparent with the antibody against the N-terminal domain of dystrophin in control sections but is absent in LV sections from the failing heart and reduced in the RV of the same heart (Fig. 1A). Amino-terminal dystrophin staining was significantly reduced in failing samples compared to normal controls (Fig. 1B). C-terminal dystrophin staining was also reduced in failing samples but to a lesser extent than the N-terminal domain (Fig. 1C).

### Table 1. Patients' Demographics and Hemodynamics Before Any Surgical Interventions

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<th>Etiology</th>
<th>Pre-Transplant/Pre LVAD Inotropes Duration (Days)</th>
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Mean ± SD.

DCM = idiopathic or familial dilated cardiomyopathy; IHD = ischemic heart disease; LVAD = left ventricular assist device; N/A = not applicable.

Figure 1. Dystrophin expression of normal and failing left ventricular (LV) and right ventricular (RV) samples. Selected immunohistochemistry for the detection of the amino (N)-terminus of dystrophin in normal control (LV and RV) and failing (LV and RV) myocardial samples (A). Staining scores for the immunohistochemical detection of N-terminus (B) and C-terminus (C) dystrophin in samples from normal controls and failing hearts.
reduced in the failing hearts with an average staining score of 1.7 ± 0.6 in LV samples and 1.9 ± 0.4 in RV samples, compared to control myocardium with an average staining score of 4.0 ± 0.5 (p < 0.001 for both ventricles: Fig. 1B). In contrast, staining for C-terminus dystrophin was indistinguishable between controls and failing myocardium (Fig. 1C).

Effect of the type of LVAD support on RV and LV remodeling. Analysis of myocardial biopsy samples from patients treated with continuous flow devices, pulsatile flow devices, and normal controls (A). Immunohistochemical detection of C-terminus dystrophin in LV and RV myocardial samples from patients treated with continuous flow devices, pulsatile flow devices, and normal controls (B). Staining scores for the immunohistochemical detection of N-terminus (C) and C-terminus (D) dystrophin in LV and RV samples from controls, failing hearts, and myocardial samples from patients treated with either continuous or pulsatile flow devices.

Figure 2. Effect of mechanical support with either a pulsatile or continuous-type left ventricular assist device on failing left ventricular (LV) and right ventricular (RV) samples. Immunohistochemical detection of N-terminus dystrophin in LV and RV myocardial samples from patients treated with continuous flow devices, pulsatile flow devices, and normal controls (A). Immunohistochemical detection of C-terminus dystrophin in LV and RV myocardial samples from patients treated with continuous flow devices, pulsatile flow devices, and normal controls (B). Staining scores for the immunohistochemical detection of N-terminus (C) and C-terminus (D) dystrophin in LV and RV samples from controls, failing hearts, and myocardial samples from patients treated with either continuous or pulsatile flow devices.
port had an effect on C-terminus dystrophin expression, which was indistinguishable from both normal and failing-heart samples (Figs. 2B and 2D; p > 0.1).

The effect of prolonged mechanical support with a continuous-type assist devices resulted in improvement on the baseline N-terminus dystrophin staining score in LV (p = 0.01) and RV (p < 0.01) samples when compared with failing hearts. However, the N-terminus still showed some disruption in the continuous flow-type LVAD-treated samples by comparison with control myocardium (Table 2). In contrast, N-terminal dystrophin staining for pulsatile-type LVAD-treated patients was statistically indistinguishable from that of the normal controls (p = 0.34; LV and RV) and significantly improved over the failing-heart samples (p < 0.001 for both LV and RV; Table 2). Interestingly, the normalization of dystrophin expression was greater in patients treated with pulsatile-type LVAD than among those treated with a continuous-type LVAD (Table 2).

**DISCUSSION**

We had previously demonstrated that LV failing myocardium was characterized by the selective disruption of the N-terminus end of dystrophin. Furthermore, we demonstrated that after prolonged LV unloading with a pulsatile-type LVAD, N-dystrophin expression normalized (7). The data presented in this article expand these previous observations by demonstrating that not only the LV but also the failing RV is characterized by selective disruption of the N-terminus of dystrophin and that unloading the LV with pulsatile or continuous flow-type LVADs led to improvements in N-dystrophin expression in left and right failing ventricles in most patients.

The importance of dystrophin in maintaining the integrity of sarcolemma and thus of the entire muscle cell during contractile stress was previously suggested by studies in mdx mice, in which dystrophin-deficient muscles show an abnormally high level of sarcolemmal rupture during muscle contraction, demonstrating a several-fold increase in susceptibility to either stretch- or contraction-induced sarcolemmal disruption when compared with normal fibers (15).

The mechanisms leading to dystrophin remodeling are unknown, but several explanations are plausible. First, the demonstration that after LV unloading dystrophin expression normalizes suggests that mechanical stress is a major mechanism for disruption. Mechanical stress imposed by myocyte hypertrophy and long-term, continuous contractile forces may create a state of fragility of the dystrophin-actin binding domain by inducing tertiary structural alterations in dystrophin. Indeed, mechanical stress results in alterations of gene expression that could lead to alterations in dystrophin expression in vivo (16,17). Second, although the improvement of RV N-dystrophin expression may be the result of hemodynamic or unloading conditions, it is also possible that improvements in the “cytokine milieu” result in deactivation of the proteases responsible for the selective disruption of N-terminal dystrophin. Indeed, it is known that the protease 2A of Coxsackievirus B3 (one of the major causes of acquired DCM) can cleave dystrophin, leading to disruption of the cytoskeleton in infected mouse hearts, suggesting this is an important pathologic mechanism in acquired DCM (18). Although in these studies, all of the myocardial samples were negative for cardiotropic viruses (enterovirus, adenovirus, CMV, EBV, and parvovirus) by PCR analysis (data not shown), it is possible that viral myocardial injury occurred before sampling (19).

Because a large portion of the failing samples and samples obtained from patients after LVAD are obtained during inotropic therapy, the question arises as to the possibility that dystrophin remodeling is influenced by either the presence or absence of inotropic therapy. germane to this discussion, it is crucial to recognize the following. When we analyzed dystrophin remodeling in patients who had received transplants without being supported by inotropes before transplant, we found decreased expression of N-terminus dystrophin just as among patients receiving inotropes. In addition, we analyzed dystrophin expression in a small subset of patients (n = 6) who were receiving inotropes at the time of LVAD implant and continued to

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<th>Table 2. Dystrophin Average Staining Scores in End-Stage Cardiomyopathy and After Mechanical Support Therapy</th>
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N-terminus score
- Failing vs. control LV: p = 0.00004
- Failing vs. control RV: p = 0.000003
- Continuous vs. control LV: p = 0.0269
- Continuous vs. control RV: p = 0.0006
- Pulsatile vs. control LV: p = 0.3370
- Pulsatile vs. control RV: p = 0.3370

LV = left ventricle; RV = right ventricle; TER = terminus.
receive inotropes until the time of transplantation. Thus, paired samples were available on patients that were constantly treated with inotropes, and in at least two of those patients there was also found an improvement on N-terminus dystrophin expression (data not shown). These data taken together provide strong evidence that the role of inotropic therapy in dystrophin remodeling is minimal at best. In the present report, we studied the effect of two modes of LVAD support with respect to their ability to modify dystrophin expression. Pulsatile-type LVADs provide a greater degree of unloading and, on full support, bypasses the aortic root and ejects directly into either the ascending or descending aorta, maintaining the pulsatility with high benefit to both myocardial oxygen balance and cardiac output (20). Continuous-type LVADs, when in full support, will not allow blood flow through the aortic valve, leading to a state of no pulsatility, and decreasing the effective systemic blood flow (21). Although full characterization of hemodynamic differences between these two forms of support does not exist, observations by our group suggest that the effect of LV unloading, as defined by the change in volumes or mass, provided by the continuous-type of LVAD may not be of the same magnitude as the one observed for pulsatile-type LVADs (G. Torre-Amione, personal communication, 2003). However, cellular regression of hypertrophy occurs in the myocardium using either continuous or pulsatile flow devices, as we recently showed by analyzing various markers in the failing RV after LVAD support (14). Additionally, the defect in dystrophin integrity is significantly ameliorated in both the left and right failing ventricle when supported with a continuous type of LVAD. Although the degree of improvement was greater for the patients supported with a pulsatile-type LVAD, it is difficult to establish definitive comparative statements, considering the small number of patients studied in this report and the variability in length of LVAD support. Indeed, although the differences were not statistically significant, there was a trend toward longer support in pulsatile-treated patients. To better address this question, we are actively involved in a multi-center LVAD study group that will allow us better definition of this and other questions of more direct clinical relevance.

A limitation of the present study comes from the fact that it was not possible to obtain intra-individual LV and RV samples before and after therapy. However, in our previous study, we demonstrated a similar pattern for dystrophin remodeling, as we showed in the present report in intra-individually LV pre- and post-LVAD biopsies. Therefore, we believe that in the present study, we demonstrate the reliability of N-terminal dystrophin detection as a marker for both LV and RV ventricular remodeling.

There are two clear important observations from these studies: 1) that the failing RV is also characterized by selective disruption of N-dystrophin, which is normalized after prolonged mechanical unloading; and 2) that there does not appear to be a significant difference in the cellular response of the myocardium to pulsatile or continuous flow types of support. These findings may be of crucial importance when considering the potential applications of LVADs in the future. For example, when one considers the possibility of myocardial recovery after LVAD support, the mode of support and the cellular changes induced by the type of LVAD on failing myocardium may determine the rate or the magnitude of recovery. One could argue that for full myocardial recovery, normalization of dystrophin expression in the left and right failing ventricle may be a prerequisite.

In summary, we have shown that, in patients with end-stage cardiomyopathies, the selective defect in the N-terminus end of dystrophin is present in both RV and LV and that this defect is reversible in both chambers after prolonged mechanical circulatory support with either a pulsatile or continuous-type of LVAD. These findings suggest that the link from sarcomere to sarcolemma and ECM is essential for the retention of normal ventricular size and function, that the dystrophin-actin binding domain is possibly fragile, and that the analysis of dystrophin-actin binding domain is important to the understanding of changes that occur with mechanical unloading.

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REFERENCES


