Muscle LIM protein deficiency leads to alterations in passive ventricular mechanics

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Omens, Jeffrey H., Taras P. Usyk, Zuangjie Li, and Andrew D. McCulloch. Muscle LIM protein deficiency leads to alterations in passive ventricular mechanics. Am J Physiol Heart Circ Physiol 282: H680–H687, 2002. First published October 25, 2001; 10.1152/ajpheart.00773.2001.—Accumulating evidence indicates that cytoskeletal defects may be an important pathway for dilated cardiomyopathy and eventual heart failure. Targeted disruption of muscle LIM protein (MLP) has previously been shown to result in dilated cardiomyopathy with many of the clinical signs of heart failure, although the effects of MLP disruption on passive ventricular mechanics and myocyte architecture are not known. We used the MLP knockout model to examine changes in passive ventricular mechanics and laminar myofiber sheet architecture. Pressure-volume and pressure-strain relations were altered in MLP knockout mice, in general suggesting a less compliant tissue in the dilated hearts. Transmural laminar myocyte structure was also altered in this mouse model, especially near the epicardium. A mathematical model of the heart showed a likely increase in passive tissue stiffness in the MLP-deficient (−/−) heart. These results suggest that the disruption of the cytoskeletal protein MLP results in less compliant passive tissue and concomitant structural alterations in the three-dimensional myocyte architecture that may in part explain the ventricular dysfunction in the dilated heart.

cytoskeleton; mechanotransduction; myofiber; pressure-volume; strain

DILATED CARDIOMYOPATHY is characterized by reduced systolic function (24) and ventricular dilation (25). Contractile dysfunction of the myocardium may be due to several factors, for example, loss of myocyte shortening capability, calcium dysregulated intracellular calcium handling, apoptosis, and ventricular geometric changes. It has been suggested that myocyte cytoskeletal defects may play an important role in the pathogenesis of dilated cardiomyopathy and heart failure (6, 14). An animal model of dilated cardiomyopathy has been described by Arber et al. (1), in which targeted disruption of the cytoskeletal component muscle LIM protein (MLP) leads to cardiomyocyte architectural disorganization through irregularities in the actin-based cytoskeletal structure. It was suggested that the structural integrity of the cardiomyocytes was compromised in this mouse model, preventing normal mechanical transduction external loads, leading to a dilated cardiomyopathic state. While several different factors have been attributed to the onset of the disease in humans (4), including a possible role of MLP abnormalities in human heart failure (30), this mouse model does reproduce many of the clinical features of human dilated cardiomyopathy and heart failure. Systolic performance of the tissue was decreased significantly in MLP-deficient mice in terms of fractional shortening and velocity of shortening of the left ventricular (LV) wall. The minimum and maximum rates of change of LV pressure were reduced and LV end-diastolic pressure was increased, consistent with LV pump failure in humans.

Although abnormal systolic performance is a hallmark of dilated cardiomyopathy, diastolic function is an important factor in overall cardiac performance and is known to change in this disease. Diastolic function in dilated cardiomyopathy may be altered by changes in the extracellular matrix; replacement and interstitial fibrosis occur in up to 20% of the ventricle (3). Diastolic filling abnormalities have been found in the disease (24), and diastolic chamber and tissue stiffness are frequently altered in the failing human heart (15). The goal of the present study was to investigate the possible role of MLP in regional passive mechanics in hearts with dilated cardiomyopathy. We hypothesized that lack of MLP in myocytes would result in structural remodeling and possible alterations in passive tissue function.

METHODS

The mice used in this study were obtained from a University of California-San Diego (UCSD) colony of MLP-deficient (MLP−/−) mice and their control littermates (MLP+/+ mice), aged 3–6 mo (1). Previous studies have shown that all surviving MLP-deficient mice develop dilated cardiomyopathy after 4–8 wk (1). The protocols for arresting the heart have been given previously (23) and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal protocols were approved by the UCSD Animal Care Subjects Committee. Each mouse was anesthetized with 8 mg/kg xylazine, 100 mg/kg ket...
amine, and 2.5 mg/kg morphine intraperitoneally. The mice were intubated and ventilated with air. The chest was opened, the ascending aorta was clamped, and the heart was arrested by slowly injecting up to 0.3 ml of a hypothermic, hyperkalemic cardioplegic solution via a stab wound through the apex into the LV. The arresting solution contained (in g/l) 4.0 NaCl, 4.4 KCl, 1.0 NaHCO3, 2.0 glucose, and 3.0 2,3-butanedione monoxime (BDM). Heparin (10,000 U/l) was also added to the solution. The arrested heart was excised and rinsed in cold saline.

Pressure-volume relationships. Pressure-volume relationships were determined by inflation of a balloon placed in the LV of the arrested heart (23). A total of five MLP−/− and five MLP+/+ control littermates were used for this part of the study. The left atrium was removed, and a balloon attached to a cannula was inserted through the mitral orifice. The balloon was connected to a Statham P23Db pressure transducer and a Harvard infusion pump with a 100-μl glass syringe. The balloon was inflated to a volume slightly less than the unloaded volume of the LV. The same size of balloon was used for all animals, and the volume of each empty balloon was recorded. The passive LV was inflated at a constant rate of 25–35 μl/min until the pressure in the LV was 25 mmHg, followed by a deflation to the original volume. Each heart was preconditioned with at least two infusion cycles until the pressure-volume curves were repeatable. Pressure and volume data were recorded at 25 Hz with an analog-to-digital conversion on a PC. For each heart, a third-order polynomial was fitted to the loading portion of the pressure-volume curve. Volumes include the residual volume of the empty balloon. From the fitted curves, volumes were averaged at 5-mmHg pressure increments for each group of hearts.

Two-dimensional surface strain. On the basis of previous experimental techniques in the rat (10, 20), two-dimensional epicardial strains during passive inflation were measured. A set of three markers was placed on the free wall of the LV using titanium dioxide powder. The marker locations were recorded onto videotape with a black-and-white video camera during an inflation cycle. The plane of the markers was approximately in the plane of focus of the camera. After the inflation cycle, the long axis of the heart was recorded and used to determine the cardiac coordinate system. The centroids of each marker were determined with an image analysis program (NIH Image) at 1-Hz intervals. On the basis of the two-dimensional locations of the markers, homogeneous strains were found on the epicardial surface using the unloaded, zero-pressure state as reference (20). Thus three independent strain components (E) were found as functions of pressure: circumferential (Eθ), longitudinal (Ez), and shear (Eφ). Strains were found as functions of volume first and then fitted to third-order polynomials, from which pressure-strain data at equal pressures in each heart could be found and averaged for each group.

Histology. To obtain accurate histological sections, separate groups of hearts were used for this purpose. Hearts were arrested in six MLP−/− and six MLP+/+ mice, excised, and perfusion fixed with buffered 10% formalin under zero ventricular load. Each heart was cut into three sections for quantification of the local myocardial fiber and “sheet” structures (8, 16) near the equator of the LV free wall. Each tissue block was embedded in paraffin; one block was sectioned parallel to the longitudinal-radial (2-3) surface, one block was sectioned parallel to the circumferential-radial (1-3) surface, and the third block was sectioned parallel to the epicardial surface (1-2) for fiber angle. These sections (10 μm) revealed laminar tissue structures separated by gaps, or “cleavage planes,” which represent projections of three-dimensional laminar fiber bundles, or sheets of myocardium, intersecting the orthogonal cardiac-coordinate planes of the ventricular wall. Once the three angles were measured, the local sheet angle was computed with a least-squares fitting procedure (8) to produce sheet and fiber angles as a function of wall depth.

The collagen area fraction was measured in the plane of the muscle. Sections (10 μm) cut parallel to the LV free wall epicardial surface were stained with trichrome, and, under bright-field microscopy, the collagen area fraction was determined using hue separation (red/orange = muscle; green/blue = collagen) computer image analysis with NIH Image at three transmural locations (epicardial, midwall, and endocardial) and averaged for each heart.

Statistics. Differences in geometric quantities and weights between MLP−/− and MLP+/+ mice were compared using an unpaired t-test. Variations in pressure-volume and pressure-strain relations were analyzed with a two-factor repeated-measures analysis of variance (variance was the within factor). A one-factor analysis of covariance was used to test for differences in fiber and sheet angles, with depth being the regressor and MLP status the between factor.

Finite-element model. To investigate the role of fiber architecture and material properties on diastolic mechanics, we developed a mathematical model of normal and genetically altered LVs, incorporating the measured muscle orientation, and predicted regional passive mechanics with each computer model.

The LVs at end diastole were modeled as truncated ellipsoids of revolution with the finite-element technique (7), with a different model for normal and dilated hearts. The geometry of the ellipsoid was set to match the average experimental data found in this study. The endocardial finite-element nodes at the base had the prolate spheroidal coordinates Θ, M, and Λ fixed to simulate the constraint of the relatively stiff mitral valve annulus (12), and the longitudinal coordinate M was fixed at all apex nodes. The symmetrical high-order finite-element model required three elements and eight nodes for convergence of the strain energy to within 0.5%.

The stress-strain relationship of the passive LV was defined by the following exponential strain-energy function W, which treats the myocardium as nonlinear, orthotropic, and nearly incompressible (28)

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W = C(e^q - 1)/2 + C_{comp}(J \times \ln J - J + 1)
\] (1)

with \(Q = b_0 E_0^2 + b_1 E_1^2 + b_2 E_2^2 + b_3 (E_0^2 + E_1^2)
+ b_4 (E_0^2 + E_1^2) + b_5 E_3^2 + E_4^2
+ b_6 E_5^2 + E_6^2),\) where the indexes f, s, and n refer to an orthogonal coordinate system having fiber, sheet, and sheet-normal axes, respectively; J is the determinant of the stretch tensor; b are constants determining the relative contribution of each strain component to the strain energy; C is the overall stiffness parameter of the material; and \(C_{comp}\) is the compressibility coefficient. The material constants were \(C = 0.88 \text{kPa}, b_{f} = 6.0, b_{s} = 7.0, b_{n} = 3.0, b_{p} = 12.0, b_{p} = 3.0, b_{n} = 3.0,\) and \(C_{comp} = 3.0.\) This strain-energy function describes the properties of myocardial tissue in local fiber-sheet coordi-
nates, taking into account actual fiber and sheet architecture.

The model was first used to predict the effect of altering fiber-sheet architecture on passive tissue strain. We subsequently used the model to examine the possible role of overall tissue stiffness by changing the overall stiffness parameter in the model and comparing these results to those from experiments.

RESULTS

A total of 22 hearts were used in this study: 10 hearts for the mechanical tests (5 MLP−/− and 5 MLP+/+ controls) and 12 hearts for the histological analysis (6 MLP−/− and 6 MLP+/+ controls). Upon gross examination, the hearts of the MLP−/− mice were larger in size, as expected, with both enlarged atria and ventricles. The hearts from MLP−/− animals were substantially heavier, and dilation of the LV was evident from the increases in inner and outer radii (Table 1).

Myofiber architecture. Myofibrillar disarray has been shown to be associated with dilated cardiomyopathy; hence, myocyte organization at the tissue level may be altered in this animal model. Measured muscle fiber and cleavage plane angles are shown in Fig. 1 relative to the wall depth for each heart. The in-plane fiber angle (1-2 plane) shows the expected trend with depth for both groups. The variation in fiber angle was essentially linear from the endocardium to epicardium in each animal, although several animals show a sigmoidal-shaped curve, as previously reported in dogs (26). Transverse cleavage plane angles were consistent within groups, with greater variations near the endocardium, as expected. Although the 1-3 angle did not appear to be different between the groups, there was a distinct change in the 2-3 angle on the outer half of the ventricular wall. Figure 2 shows the average fitted fiber angle and the computed sheet angle for the two groups. When fiber angles were fitted with linear regression and averaged, the differences in angle at the endocardium and epicardium between the groups was ~20°. Mainly because of the differences in the 2-3 angle, the sheet angle was also different in the dilated hearts at the epicardium (P = 0.032). The shift indicates sheets oriented more toward the local radial direction in the MLP-deficient hearts.

Collagen area fraction. The collagen area fraction was increased in the MLP-deficient hearts, as expected, although the greatest increases appeared to be on the epicardium and around large blood vessels. The average area fractions were 2.1 ± 0.8% in the control hearts and increased to 4.4 ± 1.3% in the dilated hearts (P < 0.02). There were no transmural differences in area fractions in either group.

Pressure-volume and strain-pressure relationships. Only the loading (infusion) portion of a single cycle was included in the mean results due to the existence of hysteresis in the pressure-volume curves. The mean time after cardiac arrest of the runs used in the pressure-volume analysis for all of the hearts was 9 ± 4 min. As expected for the dilated hearts, the unloaded volume of the LV was greater (Fig. 3). The slope of curve tended to be greater for the MLP−/− hearts, and analysis of variance showed a significant effect of treatment on the volume (P = 0.048). When each individual
pressure-volume curve was normalized to its unloaded volume, there was a significant effect of MLP on the pressure-volume relationship (interaction, \( P = 0.041 \)).

To quantify changes in regional tissue mechanics in this animal model, local epicardial strains were found as the hearts were inflated. Figure 4 shows the mean experimental results for the three epicardial cardiac strain components as functions of pressure for normal and MLP-deficient hearts. There was a significant effect of MLP disruption on passive surface function. Both longitudinal and shear strains were decreased in the MLP-deficient hearts (\( P = 0.046 \) and 0.049), and the effect of treatment on the strain-pressure relationship (interaction) was significant for the longitudinal component (\( P = 0.005 \)). The treatment did not affect circumferential strain. To estimate the effect of MLP deficiency on local fiber mechanics, the cardiac strains were rotated through the local epicardial fiber angle to obtain “fiber” strains (Fig. 5). In this case, the epicardial fiber angle (\(-72^\circ\) for MLP\(^{+/+}\) and \(-50^\circ\) for MLP\(^{-/-}\)) determined from linear regression to the histological data was used to rotate the average cardiac strains of the data shown in Fig. 4 at a pressure level of 20 mmHg. In fiber coordinates, the greatest differences were seen in cross-fiber and shear components of deformation.

**Modeling the effect of sheet orientation on strain.** The finite-element model was used to examine the effect of altered sheet orientation on passive regional function. For control hearts, coefficients for the orthotropic strain energy function were taken from previous models of the LV (28, 29). The finite-element model was passively loaded with pressure increments up to a maximum of 20 mmHg. At each load step, the mathematical model converges, giving the stress distribution in the ventricular wall that is in equilibrium with the internal pressure (boundary condition). The constitutive equation (Eq. 1) was used to find the stresses as a function of the strain components. Hence, local strains were determined from the converged model as a function of pressure. These model results show good agreement with the experimental findings (Fig. 6, MLP\(^{+/+}\)). To build a model of MLP-deficient hearts, the geometry and fiber-sheet architecture were changed according to

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Fig. 2. Dependence of mean fitted fiber (A) and sheet angles (B) on the wall for normal (MLP\(^{+/+}\)) and MLP-deficient (MLP\(^{-/-}\)) hearts. There was a small shift in the fiber angles, but sheet angle showed a large difference near the epicardium (Epi). Endo, endocardium.

Fig. 3. Pressure-volume relationship (A) and pressure-normalized volume relationship (B) for both groups. \( V/V_0 \), volume divided by unloaded volume. There was a shift in the unloaded volume, as expected for the enlarged hearts (MLP\(^{-/-}\)), and a decrease in chamber compliance in the myopathic hearts.

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the measured values. Incorporating only the changes in geometry and sheet orientation yielded model results that differed significantly from the measured strains (Fig. 6, MLP\textsuperscript{-/-}; model 1). On the basis of changes in the pressure-volume relationship for both groups (see Fig. 3), MLP-deficient hearts appear to be stiffer than normal hearts, consistent with the increased collagen area fraction. Thus we increased the coefficient $C$ in the strain-energy function by a factor of 10 to increase the overall stiffness of the material and obtained model strains that more closely matched the MLP\textsuperscript{-/-} functional results (Fig. 6, MLP\textsuperscript{-/-}; model 2).

**DISCUSSION**

The passive mechanics of the myocardium are influenced by several factors (19), including geometry, material properties, and boundary conditions. The material properties of the passive myocardium reflect the structure at the cellular level of both myocytes and the extracellular matrix. It is well known that the material properties of the myocardium depend on the local orientation of myocytes (13, 28), which have material anisotropy, and are a major determinant of passive stiffness. In the current study, we found significant alterations in the passive mechanics of the MLP-deficient heart and also structural changes in myocyte architecture. This suggests an interaction between the structural remodeling and functional abnormalities due to the lack of MLP.

Although there are limited data on the mechanical role of MLP, it has been implicated in the mechanotransduction pathway of cardiac hypertrophy and may play a role in dilated cardiomyopathy (6). It is a striated muscle-specific protein that acts to enhance protein-protein interactions at the actin-based cytoskeleton and also along the z lines of the sarcomere and is thought to be important in contractile filament integrity and cytoskeletal-membrane interactions via $\beta$-spectrin and $\alpha$-actinin (11). Thus myocytes lacking this protein would be expected to have some structural defects and possibly an altered cytoskeletal-mediated load response. We propose that an altered hypertrophic response leads to the abnormal tissue remodeling and changes in the three-dimensional myocyte structure. The normal myocyte patterns in the ventricles may be mediated via individual cell responses to external loads (2); hence, changes in cellular mechanotransduction could lead to altered global myocyte structure.

The passive pressure-volume and pressure-strain relationships have been shown to change in response to several interventions in small animal models. For example, myocardial ischemia (22), hypertrophy (9, 21), and changes in the extracellular matrix (18, 23) all lead to altered mechanical properties of the LV. Thus structural alterations at both the cellular and tissue levels correlate with altered passive mechanics. An increase in the stiffness of the myocardium in MLP-deficient mice is suggested by the experimental and numerical results of the study. If the stiffness of the tissue is indeed increased, changes in myocytes or the

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**Fig. 4.** Experimental pressure-strain relations in normal (MLP\textsuperscript{+/+}) and MLP\textsuperscript{-/-} hearts. There were significant changes in the longitudinal ($E_{22}$; B) and in-plane shear ($E_{12}$; C) independent strain components with disruption of MLP. The shear strain became essentially zero, indicating a substantial change in torsion in the dilated hearts. A: circumferential independent strain component ($E_{11}$).

**Fig. 5.** Epicardial fiber strains at 20 mmHg of pressure. Strains were found by rotating the mean cardiac strain tensor through the mean epicardial fiber angle for each group. Lack of MLP resulted in large changes in cross-fiber strain and fiber/cross-fiber shear.
extracellular matrix could be responsible at the cellular level. The abnormal actin-based myofibrillar organization associated with the lack of MLP (1) may directly affect the myocyte passive material properties. This model shows disruption in the normal z line architecture, which could relate to cross-fiber material properties. This model also produces increases in interstitial fibrosis (1), which is another possible mechanism for decreased tissue compliance. Another possible and more likely mechanism for the increase in compliance is tissue remodeling during development. Because the cytoskeleton has been implicated in mechanotransduction (6) and the MLP defect leads to cardiac dilation, there are probably profound structural remodeling responses to the altered mechanotransduction pathways, possibly leading to compensatory changes in myocyte or extracellular structure and function.

The three-dimensional nature of myocyte laminar structure has been well documented in larger species (8, 16) and seems to be conserved in the mouse. The fiber and sheet angle distributions found in the present study for control animals are very similar to those reported in the dog (8, 17). The transmural variation in the fiber angle presumably functions to optimize diastolic and systolic mechanics, as proposed in three-dimensional models (5, 13, 27). Transverse shearing along sheets is thought to provide a mechanism for normal wall thickening during systole (17). Thus the change in sheet angle with dilation may be a mechanism of reduced wall thickening. The passive mechanics of the LV are also dependent on the fibrous tissue structure (13) and sheet structure (28), although the effects of sheet structure on the passive mechanics have not been well defined. It might be expected that interlaminar sheet shearing could provide a mechanism for wall thinning during diastolic filling, similar to the effect during systole. Thus in a diseased state with altered sheet orientations, these shearing mechanisms can be affected, which in turn will alter the overall diastolic filling function.

The modeling analysis presented here is limited by the lack of knowledge of material properties of the mouse myocardium. Even in larger species, very few analyses include the laminar sheet structure in the

Fig. 6. Comparison of mean experimental strains (points) with model predictions (lines). *Left*, MLP-positive (MLP+/+) hearts; *right*, MLP-negative (MLP−−) hearts. Model parameters for the MLP+/+ hearts were taken from published results for a similar model in normal myocardium. *Model 1* for the MLP−− hearts used the same material properties as the control but changed the sheet angles and geometry to match the measured values. *Model 2* also used a stiffer material, which shows a much better match to the measured strains.
constitutive model (28). We chose this constitutive law because it incorporates the realistic laminar ultra-structure of the tissue as measured in the present study. The material parameters are essentially unknown for the mouse heart, although the parameters in the dog have been used to estimate those in a model of the mouse heart (29). It is possible to alter the individual material constants in the strain energy function, for example, the fiber and cross-fiber terms, but because we did not have three-dimensional strain data, in the present analysis we chose not to change these terms individually based solely on epicardial strains. The lack of knowledge of material properties and simple geometry used to model the LV are possible reasons for worse agreement between experimental and model data in the MLP+/− heart for $E_{12}$ and $E_{22}$ compared with $E_{11}$ (Fig. 6, left). We did modify the overall stiffness constant $C$ based on known changes of the extracellular matrix and found this change produced more favorable passive deformations of the model. In the future, mouse magnetic resonance imaging tagging will permit deformations to be measured throughout the heart; hence, more realistic analyses of the material law can be performed, which should improve the match between experimental and model results.

By examining the mechanics of the adult heart that already has developed heart failure, we were unable to directly distinguish between the effect of MLP deletion per se or the resulting heart failure. With this particular protein deficiency, most of the young animals (<2 wk of age) lacking MLP do not show any signs of heart failure. They later go on to develop heart failure after several months. It may be possible to study these young animals with MLP disruption to directly examine the effects of the missing protein, without the confounding complications of heart failure. For example, there may be direct structural effects of the missing protein on passive or active mechanics in the ventricle in addition to the putative role in mechanotransduction. The direct role of MLP in mechanotransduction is best studied in cell culture, for example, the altered response of MLP−/− cells to stretch. Alternatively, in the intact heart, other models of heart failure (for example, hypertrophy or ischemia) could show similar changes in passive structure and function, pointing to a more generic role of heart failure in this type of tissue remodeling. Future studies should be able to make this distinction.

The number of animals used in the current study was small ($n = 5$ or 6 animals/group); thus the statistical power is not as high as it would be with a greater number of animals. For the majority of significant statistics, the statistical power was good (>0.8), but in some cases the power was only in the range of 0.6–0.8, which indicates that the statistical power of the tests could be strengthened by increasing the number of animals (assuming the means and SD do not change with the greater sample size). The power for the tests with nonsignificant results was low, indicating that we cannot rule out the possibility that these parameters were not different also (for example, the circumferential strain).

On the basis of the present results, disruption of the MLP component of the myocyte cytoskeleton leads to altered passive mechanical properties of the LV that accompany the known dilation and systolic dysfunction. These functional differences exist in association with structural changes in the laminar sheet architecture in the LV that are suggestive of a possible mechanism behind the altered passive filling function. The passive dysfunction may be an important part of the overall loss of ventricular performance seen in this model of dilated cardiomyopathy.

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