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Myofilament Degradation and Dysfunction of Human Cardiomyocytes in Fabry Disease

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Early detection of myocardial dysfunction in Fabry disease (FD) cardiomyopathy suggests the contribution of myofilament structural alterations. Six males with untreated FD cardiomyopathy submitted to cardiac studies, including tissue Doppler imaging and left ventricular endomyocardial biopsy. Active and resting tensions before and after treatment with protein kinase A (PKA) were determined in isolated Triton-permeabilized cardiomyocytes. Cardiomyocyte cross-sectional area, glycosphingolipid vacuole area, myofibrillarolysis, and extent of fibrosis were also determined. Biopsies of mitral stenosis in patients with normal left ventricles served as controls. Active tension was four times lower in FD cardiomyocytes and correlated with extent of myofibrillarolysis. Resting tension was six times higher in FD cardiomyocytes than in controls. PKA treatment decreased resting tension but did not affect active force. Protein analysis revealed troponin I and desmin degradation products. FD cardiomyocytes were significantly larger and filled with glycosphingolipids. Fibrosis was mildly increased compared with controls. Tissue Doppler imaging lengthening and shortening velocities were reduced in FD cardiomyocytes compared with controls, correlating with resting and active tensions, respectively, but not with cardiomyocyte area, per-

centage of glycosphingolipids, or extent of fibrosis. In conclusion, myofilament degradation and dysfunction contribute to FD cardiomyopathy. Partial reversal of high resting tension after pharmacological PKA treatment of cardiomyocytes suggests potential benefits from enzyme replacement therapy and/or energy-releasing agents. (Am J Pathol 2008; 172:1482–1490; DOI: 10.2353/ajpath.2008.070576)

Fabry disease (FD) is an X-linked lysosomal storage disorder caused by the deficiency of the enzyme α -galactosidase A, resulting in progressive intracellular glycosphingolipid deposition in multiple organ systems, including the heart.¹ In patients with FD, cardiac involvement is characterized by progressive left ventricular (LV) wall thickening, mimicking hypertrophic cardiomyopathy,^{2,3} with diastolic LV dysfunction and a preserved LV ejection fraction that may decline in the end stage of the disease.⁴ The diastolic LV dysfunction has usually been ascribed to myocardial fibrosis in addition to cardiomyocyte hypertrophy and engulfment by glycosphingolipids. Recently however, tissue Doppler imaging (TDI) revealed reduced diastolic and systolic velocities even in the prehypertrophic phase of the disease,⁵ suggesting an early and direct involvement of cardiomyocyte function.

The present study therefore investigated in male patients with untreated FD active and resting tension of isolated cardiomyocytes, myofilament protein composition, myocardial collagen deposition, and glycosphingolipid accumulation and correlated them with TDI myocardial long axis shortening and lengthening velocities.

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Materials and Methods

Patient Population

From January 1996 to July 2005, 12 consecutive male patients with LV hypertrophy were diagnosed to have FD by means of biochemical, genetic, and endomyocardial biopsy studies. Eight patients had not yet begun enzyme replacement therapy, six of them (47.1 ± 8.3 years) had a complete clinical, morphometric, and force measurement evaluation and constituted our patient population (Table 1). The patients belonged to unrelated families. Reduced peripheral blood α -galactosidase A activity was detected in all patients as previously described⁶ and causal mutations were identified by direct sequencing of α -galactosidase A gene in all families. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Clinical Studies

Extensive clinical examination, including the assessment of FD systemic manifestations, electrocardiography, two-dimensional echocardiography with Doppler analysis, and cardiac magnetic resonance imaging (MRI) with late gadolinium enhancement, were performed in all patients.

TDI analysis was performed in the pulsed Doppler mode to record mitral annulus velocities at septal and lateral corners.^{5,7} Systolic (Sa), early diastolic (Ea), and late diastolic (Aa) velocities were measured and the E/Ea ratio was computed. Maximal wall thickness was defined as the greatest thickness of any segment of the LV wall. Ten age-matched men with no evidence of LV hypertrophy or cardiac and systemic disease were used as controls. MRI was performed as previously described.⁷ Late enhancement assessment was performed 10 to 15 minutes after injection of gadolinium-DTPA (Shering AG, Berlin, Germany) (0.2 mmol/kg of body weight), by using a three-dimensional inversion recovery T1-weighted sequence.

Cardiac Catheterization and Endomyocardial Biopsy

All invasive studies were approved by the ethical committees of our institutions and the patients provided written informed consent. All FD patients underwent coronary and biventricular angiography with biventricular or LV endomyocardial biopsy. LV end-diastolic pressure >16 mmHg was considered as indicative of LV diastolic dysfunction. Eight to ten endomyocardial samples, ~ 3 mm³ each, were obtained

Table 1. Characteristics of Fabry Disease Patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)	50	46	53	58	41	35
Cardiac manifestations*	Dyspnea, chest pain, A	Dyspnea, chest pain, A	Dyspnea, chest pain, A	Dyspnea, chest pain, A	Dyspnea, chest pain, A	Dyspnea, chest pain, A
Extracardiac manifestations†	Skin, CNS, ears, eyes, kidneys, H	Skin, CNS, ears, eyes, kidneys, AP, H	Skin, eyes, kidneys, H	Eyes, ears, kidneys	Skin, eyes, ears, kidneys	Skin, CNS, eyes, ears, kidneys
Enzymatic activity‡ (nmol/hour/mg of protein)	70.3 $\pm$ 9.6	20.9 $\pm$ 1.5	79.2 $\pm$ 8.5	50.2 $\pm$ 5.6	23.1 $\pm$ 2.6	15.2 $\pm$ 0.8
LVEDP (mmHg§)	26	24	21	19	20	22
Echocardiographic data¶						
LVEDD (mm)	49	46	44	43	45	40
MWT, mm	20	21	23	19	18	18.5
LVEF (%)	50	51	67	65	63	65
Fractional shortening (%)	38	43	48	44	40	47
E/A ratio	0.82	0.93	0.91	0.89	0.94	0.95
Isovolumic relaxation time (ms)	115	110	108	111	107	108
E-wave deceleration time (ms)	285	270	275	240	205	210
MRI data						
LV mass index (g/m ²)	150.3	132.0	143.2	133.4	99.9	114.1
Late enhancement (%)	6.7	6.3	4	7.4	1.6	7.9

*A, arrhythmias.

†CNS, central nervous system; AP, acroparesthesias; H, hypohidrosis.

‡Values are the mean ($\pm$ SD) results of three independent determinations on peripheral blood lymphocytes.

§EDP, left ventricular end diastolic pressure.

¶LV, left ventricular; EDD, end diastolic diameter; MWT, maximal wall thickness; EF, ejection fraction; EDP, left ventricular end diastolic pressure.

||MRI, magnetic resonance imaging.

from each patient. Five to six myocardial samples were processed for routine histological and histochemical analyses. Two samples were fixed in 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH = 7.3) and embedded in Epon resin; semithin sections were processed for Azur II staining and ultra-thin sections were stained with uranyl acetate and lead hydroxide for transmission electron microscopy.⁷ One to two endomyocardial biopsy samples were snap-frozen in liquid nitrogen and used for cardiomyocyte force measurements and protein analysis.

Morphometric Studies

Paraffin-embedded histological sections stained with Masson's trichrome were examined at $\times 400$ magnification with a reticule containing 42 sampling points (no. 105844; Wild Heerbrugg Instruments, Gals, Switzerland) to determine the percent area occupied by cardiomyocytes and by interstitial and replacement fibrosis.⁸ Cardiomyocyte cross-sectional area was computed measuring the cardiomyocyte diameter across the nucleus in 50 to 100 cells cut transversely (78 ± 14 cells; range, 58 to 97).⁹ At that level, the diameter of the perinuclear vacuoles was also measured and the percent cardiomyocyte area occupied by vacuoles was computed. In addition, endocardial thickness was determined. These measurements were performed in glutaraldehyde-fixed, Epon resin-embedded, semithin sections stained with Azur II to visualize glycolipid droplets. Images of the histological sections were analyzed using Lucia G software (version 4.82; Nikon, Tokyo, Japan). Morphometric analysis of myofibrillar area was performed on ultra-thin sections, stained with uranyl acetate and lead hydroxide. Photographic negatives of transmission electron microscopy sections were analyzed using KS-300 software (Carl Zeiss Co., Oberkochen, Germany).¹⁰ Ten surgical specimens from age-matched male patients with mitral stenosis and normal LV function were used as normal controls for morphometric measurements.

Force Measurements in Isolated Cardiomyocytes

Biopsies were stored in liquid nitrogen for up to 41 months (20.2 ± 16.7 , range, 3 to 41 months). Previous studies have shown that these samples can be used for force measurements in single cardiomyocytes.¹¹ Force measurements were performed in mechanically isolated single cardiomyocytes of the six patients at 15°C as described previously.^{11–13} The control group consisted of surgical biopsies from five age-matched male patients with mitral stenosis and normal LV end-diastolic pressure, chamber dimensions, and contractile function. Briefly, frozen biopsies were defrosted within 10 seconds in cold relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; MgATP, 4; imidazole, 10; pH 7.0). Cells were mechanically isolated and incubated for 5 minutes in relaxing solution supplemented with 0.5% Triton X-100 to remove all membranes. Thereafter, cells were washed twice in relaxing solution and a single cardiomyocyte was

Figure 1. Single FD (A) and control (B) cardiomyocyte attached between a force transducer and a piezoelectric motor. C: Contraction-relaxation sequence in FD cardiomyocyte before (left) and after (right) treatment with PKA. The abrupt changes in force mark the transitions of the preparation through the interface between solution and air (ie, transfers between wells containing relaxing and activating solutions).

attached between a force transducer and a piezoelectric motor using silicone adhesive (Figure 1, A and B). To enable attachment between the force transducer and motor single preparations were selected for measurements on the basis of cell length ($\sim 100 \mu\text{m}$ long). Resting sarcomere length of isolated cardiomyocytes was $\sim 1.7 \mu\text{m}$ and was adjusted to $2.2 \mu\text{m}$ for measurements of isometric force. The composition of the relaxing [pCa ($-\log[\text{Ca}^{2+}]$), 9.0] and activating (pCa, 4.5) solution was previously described.¹⁴ All force values were normalized for cardiomyocyte cross-sectional area. A typical contraction-relaxation sequence in a cardiomyocyte from a Fabry sample is shown in Figure 1C. After curing of the glue for 50 minutes, the cardiomyocyte was transferred from the isolating solution on the mounting area to a small temperature-controlled well (volume, $80 \mu\text{l}$) containing relaxing solution. Isometric force was measured, after the preparation was transferred, by moving the stage of the inverted microscope to a temperature-controlled well containing activating solution.¹² On transfer of the cardiomyocyte from relaxing to activating solution, isometric force started to develop. Once a steady-state force level was reached, the cell was shortened within 1 ms to 80% of its original length to determine the base line of the force transducer. The distance between the base line and the steady force level is the total force (F_{total}). After 20 ms the cell was restretched and returned to the relaxing solution, in which a second slack-test of 10 seconds duration was performed to determine resting or passive force ($F_{passive}$). The difference between F_{total} and $F_{passive}$ is the active force (F_{active}) developed by the cardiomyocyte. After measurements of F_{total} and $F_{passive}$, the cardiomyocytes were incubated for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of protein kinase A (PKA) (100 U/ml, batch 12K7495; Sigma, Brooklyn, NY) and 6 mmol/L dithiothreitol (MP Biochemicals, Irvine, CA). Subsequently, force

measurements were repeated. Control incubations in relaxing solution with 6 mmol/L dithiothreitol, but without PKA, did not alter F_{passive} and F_{active} of cardiomyocytes.

Protein Analysis

Protein analysis was performed on cardiomyocytes, which were not used for force measurements. After isolation and Triton treatment the remaining cell pellet was freeze-dried and homogenized in sample buffer. To detect myofilament proteolysis, myofilament proteins were separated by one-dimensional gel electrophoresis containing 15% total acrylamide (acrylamide to bis-acrylamide ratio, 37.5:1) followed by Western immunoblotting.¹⁵ Five μg (dry weight) of the tissue samples were applied to the gels. Western immunoblot analysis was performed using specific monoclonal antibodies against troponin I (TnI) (clone 8I-7, dilution 1:1000; Spectral Diagnostics Inc., Toronto, Ontario, Canada), desmin (clone DE-U-10, dilution 1:1000; Sigma), myosin light chain 1 (clone F109.16A12, dilution 1:200; Alexis Biochemicals), myosin light chain 2 (clone F109.3E1, dilution 1:200; Alexis Biochemicals, San Diego, CA), and α -actinin (clone EA-53, dilution 1:1000; Sigma) and signals were visualized using a secondary horseradish peroxidase-labeled goat-anti-mouse antibody and enhanced chemiluminescence (ECL Plus Western blotting detection; Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Normal distribution of variables was assessed with Kolmogorov-Smirnov and Shapiro-Wilk tests. Variables showing normal distribution are presented as mean $\pm$ SD. Variables not showing normal distribution are presented as median (interquartile range). Categorical variables are presented as proportions or percentages. Continuous variables, showing a normal distribution, were compared with Student's *t*-test for independent samples (cases versus controls). Continuous variables not showing a normal distribution were compared with Mann-Whitney test (cases versus controls). Bivariate correlations were analyzed by Spearman rho coefficient computation. A two-tailed $P < 0.05$ was considered statistically significant. Statistical analysis was performed with SPSS version 11.0.1 software (SPSS Inc., Chicago, IL).

Results

Clinical Studies

Patients' clinical characteristics, echocardiographic and MRI data are reported in Table 1. α -Galactosidase A activity was very low (mean value, 43.4 ± 7.4 nmol/hour/mg of protein; normal range, 3252 to 1623 nmol/hour/mg of protein) and all patients had extra-cardiac clinical manifestations of the disease. All patients were normotensive, satisfied the electrocardiographic and echocardiographic criteria for LV hypertrophy, and showed an increase in LV mass index. Diastolic function

Figure 2. **A:** Cardiac MRI short (left) and long (right) axis view showing basal infero-lateral hyperenhancement in FD patient 3 of Table 2. **B:** LV endomyocardial biopsy from the same patient showing mild interstitial and focal replacement fibrosis (F) and severe glycolipid engulfment of cardiomyocytes (Masson staining). **C:** Volume composition of the myocardium in endomyocardial biopsies of FD compared with controls revealing no significant difference in percentage of cardiomyocytes (M) and other interstitial components (O), whereas interstitial (IF) and replacement (RF) fibrosis showed a mild significant increase. Results are mean $\pm$ SD. * $P < 0.05$ compared with controls. Original magnification, $\times 40$ (**B**).

was impaired in all patients but no restrictive filling pattern was detected. Conversely, systolic function, as measured by ejection fraction and fractional shortening, was within the normal range in all FD patients.

Gadolinium contrast-enhancement MRI study showed late enhancement in all patients typically localized in the basal or basal-medium segment of the lateral and infero-lateral wall (Figure 2A). Two patients (Table 1, patients 2 and 6) showed additional focal late enhancement in the apex. The mean percentage of myocardium involved was $5.7 \pm 2.4\%$ (range, 1.6 to 7.9). On TDI examination all patients had significant reduction of long axis lengthening and shortening velocities measured at the septal and lateral corner of the mitral annulus (Table 2). LV angiography revealed normal wall motion in all patients and coronary angiography showed absence of significant coronary stenoses.

Table 2. TDI, Morphometric and Force Measurements in Patients with Fabry Disease Compared with Controls

	Fabry disease	Controls*	P†
TDI velocities			
Septal Sa, cm/second	5.9 ± 0.4	14.6 ± 1.2	<0.001
Septal Ea, cm/second	5.7 ± 0.5	15.5 ± 1.3	<0.001
Septal Aa, cm/second	6.0 ± 0.5	10.3 ± 0.9	<0.001
Septal E/Ea	11.2 ± 0.5	6.0 ± 1.3	<0.001
Lateral Sa, cm/second	6.0 ± 0.2	13.7 ± 2	<0.001
Lateral Ea, cm/second	5.9 ± 0.4	14.0 ± 0.4	<0.001
Lateral Aa, cm/second	6.1 ± 0.6	10.1 ± 1.5	<0.001
Lateral E/Ea	10.5 ± 0.3	5.8 ± 1.2	<0.001
Morphometric data			
Fibrosis, %	8.7 ± 4.4	4.1 ± 2.5	0.02
Interstitial	6.7 ± 3.1	3.4 ± 2.0	0.02
Replacement	1.9 ± 1.5	0.7 ± 0.6	0.03
Cardiomyocytes, %	81.2 ± 7.2	88.1 ± 6.1	0.06
Cardiomyocyte area, μm^2	861.0 ± 208.4	215.4 ± 90.1	<0.001
Cardiomyocyte area occupied by vacuoles, %	57.3 ± 4.1	0	<0.001
Myofibrillolysis area, %	15.2 ± 4.9	0	<0.001
Force measurements†			
F _{total} , kN/m ²	13.7 ± 3.9‡	17.6 ± 5.3	NS
F _{active} , kN/m ²	3.9 ± 3.5	15.9 ± 5.4	<0.001
F _{passive} , kN/m ²	9.8 ± 2.3‡	1.6 ± 0.6	<0.001
F _{total} after PKA, kN/m ²	11.1 ± 2.6	18.2 ± 5.4	0.02
F _{active} after PKA, kN/m ²	4.3 ± 3.3	16.8 ± 5.4	<0.001
F _{passive} after PKA, kN/m ²	6.6 ± 2.6	1.4 ± 0.6	<0.001

Data are present as mean value ± SD. $P < 0.05$ was considered statistically significant.

*Controls for TDI were $n = 10$, for morphometric studies were $n = 10$, for force measurements were $n = 8$.

†P values obtained comparing FD versus controls.

‡P < 0.001 versus after PKA treatment.

Histological and Morphometric Studies

Histological examination of FD endomyocardial biopsies revealed regularly arranged and severely hypertrophied cardiomyocytes with large perinuclear vacuoles containing material that, on frozen sections, stained positively with periodic acid-Schiff and Sudan black stains. Fibrosis was predominantly interstitial (Figure 2B) with focal areas of replacement fibrosis. Computer-assisted histomorphometry showed only a mild, although significant, increase in fibrosis in FD patients compared to controls. The percent tissue area occupied by cardiomyocytes was similar (Figure 2C). Cardiomyocyte cross-sectional area was significantly increased in FD patients and more than 50% of it was occupied by glycosphingolipid vacuoles. The endocardium was thickened (mean FD value = $535 \pm 201 \mu\text{m}$, normal value = $18 \pm 5 \mu\text{m}$).

On ultrastructural electron microscopic examination intracellular vacuoles appeared to be represented by concentric lamellar structures in single-membrane bound vesicles, indicative of lysosomal glycosphingolipid accumulation. The cytoplasmic inclusions frequently displaced cardiac myofibrils to the periphery of the cell (Figure 3A). Focal areas of myofibrillolysis were also detected (Figure 3B) and myofibrillolysis area was calculated as $15 \pm 5\%$. Lamellar inclusions were present also in the endothelial cells, smooth muscle cells and fibroblasts. Cardiomyocyte area and percent area occupied by glycolipid vacuoles closely correlated (correlation coefficient = 0.99, $P < 0.0001$). This indicates that the increase of cardiomyocyte size is mainly attributable to intracytoplasmic glycosphingolipids vacuoles.

Force Measurements in Isolated Cardiomyocytes and Protein Analysis

Force measurement data of six FD patients (number of cardiomyocytes = 17) and of five controls (number of cardiomyocytes = 10) are presented in Table 2 as average value and in Table 3 as single patient value, in addition to the patients' gene mutation. When isolated cardiomyocytes were stretched to a sarcomere length of $2.2 \mu\text{m}$ a significantly higher F_{passive} and a lower F_{active} was observed in FD patients compared to controls. Treatment with PKA significantly decreased F_{passive} , but it remained higher compared to controls. F_{active} was not altered (Figure 4A). PKA treatment did not alter F_{passive} and F_{active} in controls.

Analysis of myofilament proteolysis revealed degradation products of TnI and desmin in FD patients (Figure 4B). No degradation products of myosin light chains or of α -actinin were observed. Degradation of TnI was found in three of six samples and amounted on average to $3.1 \pm 1.4\%$ of total TnI (Table 3). Desmin degradation products were present in four of six samples and amounted to $9.7 \pm 5.8\%$ of total desmin (Table 3).

Correlation between TDI, Morphometric, and Force Measurements

To establish if alterations in cardiac systolic and diastolic function could be ascribed to changes in morphometry or myofilament function or a combination of both, *in vivo* measurements of cardiac function by TDI were correlated with fibrosis, cardiomyocyte area, percent area of glyco-

Figure 3. **A:** Electron microscopy showing comparison of the normal arrangement of myofilaments in a normal cardiomyocyte (**A**) and myofilament dislodgment and disarray attributable to glycosphingolipid accumulation in FD cardiomyopathy (**B**). **B:** Electron microscopy of FD cardiomyopathy showing myofibrilolysis areas (M) around glycolipid deposits (G).

sphingolipids vacuoles, and myofilament F_{active} and $F_{passive}$. The average F_{active} of each individual correlated with TDI shortening velocities at both corners of the mitral annulus (Figure 5, A and B; correlation coefficient = 0.99, $P < 0.001$, for septal Sa and 0.90, $P < 0.05$, for lateral Sa). The average of $F_{passive}$ of all cardiomyocytes of each individual correlated closely with TDI long axis lengthening velocities (Figure 5, C and D; correlation coefficient = 0.99, $P < 0.001$, for septal E/Ea and 0.94, $P < 0.05$ for lateral E/Ea). F_{active} inversely correlated with myofibrilolysis area (Figure 5E, correlation coefficient = 0.94, $P <$

Figure 4. **A:** Graph showing total (F_{total}), active (F_{active}), and passive ($F_{passive}$) force before and after PKA in FD cardiomyocytes ($P = 0.02$ total force before versus after, $P = 0.4$ F_{active} before versus after $P = 0.004$, $F_{passive}$ before versus after). **B:** Protein analysis using one-dimensional gel electrophoresis. Ponceau-stained blot of molecular weight marker (M) and myofilament proteins (P) (**1**). Degradation products of TnI (**2**) and desmin (**3**) in a control sample (nonfailing donor heart), kept at 20°C for 1 hour, and in FD samples, in which similar degradation products are evident.

0.05). After PKA treatment, $F_{passive}$ closely correlated with area of glycosphingolipid deposits (Figure 5F, correlation coefficient = 0.99, $P < 0.001$). TDI lengthening and shortening velocities did not correlate with cardiomyocyte area, percent area occupied by glycosphingolipids vacuoles, and extent of fibrosis.

Discussion

Recent studies^{5,20} using TDI imaging provided evidence for diastolic and systolic LV dysfunction in FD patients even before the development of wall thickening. The basis of these functional abnormalities, which become more prominent as LV hypertrophy progresses, is still unclear. Especially the relative contribution of myocardial fibrosis and cardiomyocyte dysfunction remains uncer-

Table 3. α -Gal A Mutations, Force Measurements, and Protein Analysis in Fabry Disease Patients

Patient	α -Gal A mutation	Force before PKA (kN/m ²)			Force after PKA (kN/m ²)			TnI degradation (%)	Desmin degradation (%)
		F_{total}	F_{active}	$F_{passive}$	F_{total}	F_{active}	$F_{passive}$		
1	M42fsX55 ¹⁶	14.9	1.2	13.7	11.4	1.5	9.9	0.0	28.6
2	D315fsX315 ¹⁶	12	1.7	10.3	11.0	1.3	9.7	0.0	0.0
3	Y216C*	9.2	2.0	7.3	9.1	3.1	6.0	7.9	2.2
4	N215S ¹⁷	15.6	5.6	10.0	13.2	6.2	6.0	5.3	27.4
5	R220X ¹⁸	10.4	2.9	7.6	7.4	3.7	3.7	0.0	0.0
6	Q279K ¹⁹	19.9	10.3	9.6	14.4	10.0	4.4	5.2	0.3

*New missense mutation.

Figure 5. Scatter plot of the correlations between active force and septal Sa (**A**: correlation coefficient = 0.99, $P < 0.001$), active force and lateral Sa (**B**: correlation coefficient 0.90, $P < 0.05$), passive force and septal E/Ea (**C**: correlation coefficient = 0.99, $P < 0.001$), passive force and lateral E/Ea (**D**: correlation coefficient = 0.94, $P < 0.05$), active force and myofibrillarolysis area (**E**: correlation coefficient = 0.94, $P < 0.05$), passive force after PKA treatment and area of glycosphingolipid vacuoles (**F**: correlation coefficient = 0.99, $P > 0.001$).

tain. The present study therefore analyzed mechanical properties of isolated cardiomyocytes, degree of glycosphingolipid accumulation, and myocardial fibrosis in endomyocardial biopsy samples of FD patients and correlated these parameters with TDI evaluation of LV function. Altered cardiomyocyte relaxation and contraction, derived mainly from myofilament degradation and dysfunction, was found to be a major determinant of FD cardiomyopathy. These preliminary data, obtained in a limited number of patients, generate new hypotheses on myocardial dysfunction of FD and open new areas of research for possible additional therapeutic strategies.

Compromise of Cardiomyocyte Contractility in Fabry Cardiomyopathy

Despite a normal LV ejection fraction on routine echocardiography, TDI and strain rate echocardiography^{5,20} demonstrated reduced contractility in FD cardiomyopathy and suggested an early global and regional systolic function deficit, progressively deteriorating in untreated patients. To clarify the mechanism of the contractile deficit we evaluated the maximal isometric tension of single cardiomyocytes and demonstrated that it was reduced. Moreover it correlated with the decreased TDI systolic velocities and with the ultrastructural evidence of myofi-

brillolysis. In addition, myofibrillarolysis was associated with degradation of myofilament proteins.

In several models of ischemia/reperfusion the decrease in maximal force of the myofilaments has been ascribed to degradation of TnI. TnI degradation may be triggered by activation of the calcium-dependent protease calpain-1²¹ or by increased preload²² and has been observed in human ischemic cardiac disease.²³ Moreover, degradation of desmin was shown to play a significant role in calpain-1-induced myofilament dysfunction²⁴ and desmin degradation has been demonstrated to correlate with reduced cardiac function in ischemic human heart failure.²⁵ Because degradation products of both TnI and desmin were observed in FD biopsies, and were paralleled by ultrastructural evidence of myofibrillarolysis, part of the reduction in cardiomyocyte contractility may be explained by proteolysis. An additional obstacle to cell contraction is represented by myofilament derangement resulting from intracellular glycosphingolipid storage that causes loss of vectorial orientation and a detrimental functional effect.

Whatever the mechanism of myofilament and cell dysfunction, they can trigger at the end, similarly to hypertrophic cardiomyopathy,²⁶ trophic stimuli leading to cell hypertrophy. Indeed, it has been recently shown in FD patients the presence of circulating growth-promoting factors²⁷ able to induce *in vitro* a hypertrophic response of cardiomyocytes. In substance, opposite biological events of myofilament degradation/dysfunction and synthesis, in attempt of structural and functional cell repair, occur in human cardiomyocytes with FD. In this regard, the elevation of electrocardiography voltages associated to disease progression is partially attributable to synthesis of contractile elements and possibly to the accumulation of glycosphingolipids intracellularly and on the plasma membrane,²⁸ affecting the intracellular resistivity to the activation wave front and the conduction velocity.

Increased Cardiomyocyte Stiffness in Fabry Cardiomyopathy

The present study showed F_{passive} of isolated cardiomyocytes to be six times higher in FD patients than in controls. *In vivo* measures of LV diastolic function, such as TDI long axis lengthening velocity correlated with the *in vitro* measurements of F_{passive} . This indicated that in Fabry cardiomyopathy diastolic LV dysfunction is related to cardiomyocyte stiffening. These data are in agreement with recent studies on diastolic heart failure, which reported involvement of cardiomyocyte stiffness in the pathogenesis of diastolic LV dysfunction.^{13,29} Because the endomyocardial collagen structure was removed and the integrity of sarcolemmal and sarcoplasmic membranes was disrupted during cardiomyocyte isolation and permeabilization, the increased F_{passive} cannot be related to modifications of sarcoplasmic proteins and/or channels, but should be ascribed to alterations in myofilament or cytoskeletal proteins.

Modifications of contractile and cytoskeletal proteins may be posttranslational and involve altered phosphory-

lation, oxidative changes, and proteolysis. Cardiac relaxation is enhanced via β -adrenergic activation of PKA and subsequent phosphorylation of myofilament proteins including TnI, myosin binding protein C, and titin. Partial correction of F_{passive} after PKA treatment suggests hypophosphorylation of these PKA target proteins, but the incomplete recovery of F_{passive} after PKA treatment implied additional intracellular mechanisms to account for the altered passive properties of the cardiomyocytes. Because glycosphingolipid deposits occupied more than half of the cardiomyocyte area and because they were organized in structurally complex concentric lamellar bodies, they probably hinder cardiomyocyte relaxation. The percent area occupied by glycosphingolipid vacuoles indeed closely correlated with increased F_{passive} after PKA treatment. Thus, our results suggest that increased stiffness of cardiomyocytes contributes to diastolic dysfunction in FD cardiomyopathy. This increased stiffness is explained both by mechanical hindrance because of the glycosphingolipid storage material and by hypophosphorylation of myofilament and/or cytoskeletal proteins.

Role of Fibrosis

Although fibrosis in endomyocardial tissue increased with progression of FD cardiomyopathy and was paralleled by expansion of cardiomyocyte area and glycosphingolipid vacuoles, its extent was only slightly increased compared with controls and did not correlate with TDI measurements of systolic or diastolic LV function. In addition, late myocardial MRI gadolinium enhancement, which is ascribed to focal fibrosis,³⁰ was mild and typically localized in the infero-lateral region of the left ventricle, with little involvement of the remaining LV segments. However, because fibrosis is unequally distributed along the LV wall, the percent detected in the endomyocardium is compatible with the presence of different, yet limited, amounts in the middle and subepicardial layers.

In summary, myocardial fibrosis does not appear to be the predominant mechanism causing diastolic dysfunction in FD cardiomyopathy with preserved LV contractility. Nevertheless, the limited number of patients studied requires to be cautious in the interpretation of these negative results and do not exclude a prominent role of myocardial fibrosis in the more advanced stages of the disease.

Clinical Implications and Conclusions

Cardiomyocyte dysfunction and structural alterations of myofilaments seem to significantly contribute to the LV dysfunction observed in FD cardiomyopathy. Prospective studies using sequential endomyocardial biopsies during enzyme replacement therapy could establish the reversibility of cardiomyocyte dysfunction and structural myofilament alteration. Partial reversal of the high cardiomyocyte resting tension after PKA could provide an inroad

for pharmacological correction of the diastolic LV dysfunction observed in FD.

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