Accumulation and Assembly of Myosin in Hypertrophic Cardiomyopathy With the 403 Arg to Gln β-Myosin Heavy Chain Mutation

Tomas Vybiral, Philip R. Deitiker, Robert Roberts, and Henry F. Epstein

The sarcomeric proteins and organization of cardiac myofibrils appeared intact in multiple unrelated patients with hypertrophic cardiomyopathy. In two subjects demonstrating the missense mutation at position 403 (Arg to Gln) in the β-myosin heavy chain gene, total myosin and immunoreactive β-myosin heavy chain levels were similar to those found in other patients with hypertrophic cardiomyopathy and various disease control subjects. No alteration in expression of the cardiac α-myosin heavy chain gene was observed. These results are consistent with the examined myosin heavy chain mutation, permitting proper accumulation and assembly of myosin while primarily impairing contractile function. The characteristic myocyte disarray would appear likely to be a secondary consequence of the mutations. (Circulation Research 1992;71:1404–1409)

Key Words • myofibrillar integrity • polarized light microscopy • immunofluorescence • human myofibrillar proteins

Familial hypertrophic cardiomyopathy (HCM) is a clinically and genetically heterogenous disorder.¹ Schier and Adelstein² have shown that the peptide structures and enzymatic activities of myosins purified from HCM and control cardiac muscles are indistinguishable from one another. Molecular genetic analyses at the DNA level, however, have led to the recent identification of mutations in the myosin heavy chain (MHC) family of genes located on chromosome 14q1 as likely causes of HCM in some families.³–⁷

In the present report, we have analyzed the organization and protein composition of myofibrils in cardiac muscle biopsies of patients with HCM, including two individuals with a missense mutation of the β-cardiac MHC gene defined at the DNA level (residue 403, Arg to Gln). HCM biopsies were similar to control biopsies with respect to myofibrillar protein content including α- and β-MHC. The observed integrity of sarcomeric A band structure is consistent with the specific β-MHC mutation resulting primarily in subclinical contractile dysfunction with hypertrophy and disarray as secondary consequences of this dysfunction.

Materials and Methods

Human Muscle Tissues

Human left ventricular and septal muscle tissue was obtained from individuals undergoing cardiac transplantation or left ventricular outflow tract myomectomy for HCM at The Methodist Hospital, Houston, Tex. The individuals were diagnosed with HCM by clinical criteria and echocardiography.⁸ Right ventricular muscle tissue was obtained from diagnostic right heart catheterization. This protocol was approved by the Human Investigations Committees of the Baylor College of Medicine and The Methodist Hospital. All HCM specimens were examined independently by a pathologist and exhibited myocyte to myocyte disarray by bright-field microscopy of hematoxylin and eosin-stained sections. Human skeletal muscle was obtained from vastus lateralis biopsy. Tissues obtained from other institutions were processed as above, transferred to our laboratory, and stored on dry ice. Immediately after removal, samples were dissected on ice, and sample aliquots were frozen at −80°C. Additional samples were embedded in Tissue-Tek OCT compound (Miles Laboratories, Naperville, Ill.) and stored at −196°C.⁹

Protein Gel Electrophoresis and Detection

Frozen specimens (approximately 0.5 g each) were embedded in OCT compound and sectioned at a thickness of 4 μm on a Minotome cryostat (IEC, Needham Heights, Mass.) at −20°C. Sectioned tissue was processed as described previously.⁹,¹⁰ Proteins were resolved by Tris-based discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were detected by Coomassie blue staining. Molecular-sized standards and their approximate Mr are as follows: human cardiac β-MHC, 223 kd; Escherichia coli β-galactosidase, 116 kd; rabbit muscle glycogen phos...
phorylase b, 98 kd; bovine serum albumin, 66 kd; actin, 42 kd; and bovine erythrocyte carbonic anhydrase, 29 kd. Quantitative transmission densitometry of the MHC (Mr, 223 kd\(^{11,12}\)) and actin (42 kd\(^{13}\)) protein bands on Coomassie blue wet-stained gels was performed on a model 620 Videodensitometer (Bio-Rad Laboratories, Richmond, Calif.) to adjust samples for equal protein content. Protein band measurements were taken in horizontal and vertical dimensions, and the cross-product values were obtained.

**Antimyosin Antibodies**

The monoclonal antibodies F88 8H8 and F88 10C2, as supernatants of hybridoma cultures, were a generous gift of Dr. Jean J. Leger, INSERM, Montpellier, France. The anti-human \(\beta\)-MHC monoclonal antibody F88 8H8 recognizes the region between Leu, 1,080 and Asp 1,096 in the human cardiac \(\beta\)-MHC sequence.\(^{11,12}\) The cardiac anti-human \(\alpha\)-MHC monoclonal antibody F88 10C2 recognizes the region about residue 1,300, located near the hinge light meromyosin junction (J.J. Leger, personal communication). For immunoblotting, monoclonal antibody F88 8H8 was used at 1:10 dilution, and monoclonal antibody F88 10C2 was used at 1:5 dilution. For immunofluorescence, the hybridoma supernatants were used undiluted.

**Myosin Heavy Chain Immunoblotting**

After resolution on a 4.5% SDS-PAGE, proteins were transferred by electrophoresis onto nitrocellulose paper as previously described.\(^9,10\) Untransferred proteins were visualized on the posttransfer gel with silver nitrate staining. Even after prolonged electrophoresis (up to 17 hours), significant quantities of MHC did not transfer. Staining of the nitrocellulose blots with 0.1% (vol/vol) indigo carmine demonstrated that some fraction of all protein species detectable on gels transferred. After transfer, the nitrocellulose blots were incubated at room temperature for 1 hour with the respective primary antibody, followed by incubation for 1 hour with a heavy chain– and light chain–specific, affinity-purified alkaline phosphatase–conjugated goat anti-mouse immunoglobulin G (Cappel-Organon Teknika Corp., Durham, N.C.) at 0.5 \(\mu\)g/ml. The blots were developed in Tris-buffered (pH 9.5) 0.033% (wt/vol) nitro blue tetrazolium and 0.016% (wt/vol) 5-bromo-4-chloro-3-indolyl phosphate until sufficient intensity was reached. Transfer experiments with double nitrocellulose membranes indicated that only minimal amounts of MHC transferred through the first membrane.

**Immunofluorescence of Skeletal Muscle Myosins**

Tissue embedded in OCT was sectioned in \(-20^\circ\)C at a thickness of 4 \(\mu\)m in a Minotome cryostat. The

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Source</th>
<th>MHC/actin ratio</th>
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<td></td>
<td></td>
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<tr>
<td>1</td>
<td>M</td>
<td>52</td>
<td>Normal LA, explant heart</td>
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<td>2</td>
<td>F</td>
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<td>Normal LVOT, explant heart</td>
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<td>VL muscle, biopsy (from same patient as specimen 14)‡</td>
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MHC, myosin heavy chain; LA, left atrium; LVOT, left ventricular outflow tract; ASHD, atherosclerotic heart disease; LV, left ventricle; HCM, hypertrophic cardiomyopathic; RV, right ventricle; VL, vastus lateralis femoris skeletal muscle.

*Familial linkage to chromosome 14q1 (M.B. Perryman and R. Roberts, unpublished results).
†Familial linkage to chromosome 14q1.\(^6\)
‡Missense mutation of \(\beta\)-MHC gene at position 403, Arg to Gln (M.B. Perryman and R. Roberts, unpublished results).
sections were placed onto silane-coated microscope slides (Histological Control Systems, New York), air dried for 10 minutes, and stored unfixed at −80°C until use. Slides were incubated with the primary antisera for 1 hour at 37°C, followed by incubation with an affinity-purified fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Cappel-Organon Teknika) at 10 µg/ml for 1 hour at room temperature.

Light Microscopy

Separate tissue sections (4 µm thick) were stained with hematoxylin and eosin and examined by polarized light in a Reichert Zetopan microscope. Tissue sections prepared by immunofluorescence were viewed in a Zeiss Photomicroscope III with fluorescein-specific filters or alternatively by phase contrast.

Results

Myofibrillar Proteins in the 403 Arg to Gln Mutant β-MHC and Other HCM

To examine the effects of a defined β-MHC mutation on associated myofibrillar proteins, we studied the protein composition of crude preparations of sectioned cardiac muscle from two subjects with HCM who had a predicted missense mutation in the β-MHC gene at position 403 (Arg to Gln) compared with diseased control tissue, including HCM with unknown molecular defects. Patient information is listed in Table 1. Individual specimens were processed according to a previously described protocol suitable for small quantities of muscle.10 Despite the samples having been stored for up to 3 years, overall protein breakdown, as assessed by densitometry of MHC and actin, appeared to be low. Once processed and stored in gel running buffer at −80°C, sample aliquots did not show any further degradation.

Figure 1C shows that the major myofibrillar proteins were represented at comparable levels in both mutant β-MHC HCM and control tissues. Densitometry of MHC and actin revealed no significant difference in their levels between the various tissue samples (Table 1, p>0.1 by analysis of variance). Although the MHC/actin ratio of the specific missense mutant was within the control range, small differences in various minor protein bands appeared consistently, as shown in lane 4 of Figure 1C.

Immunoblotting with specific monoclonal antibodies against α- and β-MHC (Figures 1A and 1B) revealed the β-MHC isoform to be predominant in atrial, septal, and free left ventricular wall biopsies of HCM with mutant β-MHC and control samples. When matched for equal MHC content, all samples exhibited equal β-MHC reactivity. Cardiac α-MHC was detected by immunoblotting only in atrial muscle in spite of overloading of the gel lanes of ventricular samples with respect to the MHC protein band (see Figure 1A).

Sarcomeric A Band Structure in the 403 Arg to Gln β-MHC Mutant and Other HCM

We analyzed cardiac intracellular structure in sections of the same tissue samples used for the protein analyses by taking advantage of the known birefringence of striated muscle due to the supramolecular organization of myosin (A bands) and actin (I bands) within the sarcomere.14,15 Polarized light microscopy revealed the regular arrangement of A and I bands in HCM that was due to a missense mutation in the cardiac β-MHC gene (Figure 2). These results were obtained routinely in unfixed samples that were frozen rapidly for biochemical analysis.

Cardiac β-MHC Within Skeletal Myofibrils

Because of limited cardiac biopsy material from the patients with the specific missense mutation, we analyzed one patient's skeletal muscle. Figure 3 demonstrates that cardiac β-MHC is expressed in skeletal
muscle fibers of a subject with the 403 Arg to Gln β-MHC mutation (Table 1, specimen 13) in a manner similar to that found in control specimens (data not shown). Similar myosin/actin ratios were observed in skeletal muscles of patients with chromosome 14q1–associated HCM and control samples (Table 1). No α-MHC reactivity was detected in skeletal muscle by immunoblotting or immunofluorescence.

Discussion

Cardiac biopsies taken from two subjects with HCM who had a predicted β-MHC mutation (403 Arg to Gln) were found to be normal in MHC composition. In these heart tissues, β-MHC was assembled into organized sarcomeres, as evidenced by a regular sarcomeric structure. Cardiac β-MHC was expressed properly in skeletal muscle of these HCM and control subjects.

Because recent reports implicate mutations in the MHC gene family as putative causes of HCM in some families, the examination of myocardial sarcomeric architecture and protein accumulation in persons with defined alleles has become particularly important. Since alteration of one sarcomeric component may influence the expression of other associated elements, we quantitated MHC and actin protein bands on SDS-PAGE by densitometry. Our results indicate that HCM samples, including samples from two subjects with a defined β-MHC missense head mutation, exhibit MHC/actin ratios similar to those found in control samples. Thus, it would appear that the clinical picture of HCM does not arise as a result of a failure of MHC or actin to accumulate in the myocyte. The specific missense mutants consistently show differences in the levels of minor protein bands. The cause of these small differences remains unknown; however, increased specific turnover rate in vivo or increased proteolysis of sarcomeric protein in vitro in the specific β-MHC mutant cannot be excluded.

Geisterfer-Lowrance et al have hypothesized that mutations in MHCs may lead to a poisoning of the assembly of myosin into myofilaments. Our findings in two HCM patients who had the 403 Arg to Gln missense mutation of the β-MHC gene demonstrate regular sarcomeric organization by polarized light microscopy. These results suggest the proper assembly of β-MHC-containing myosin molecules.

Cardiac myocyte disarray is the accepted criterion for the pathological diagnosis of HCM and is hypothesized to be closely related to its pathogenesis; however, this disarray has also been described in a variety of unrelated cardiac disorders and in apparently normal

Figure 2. Polarized light microscopy of longitudinal sections of cardiac muscle samples. Regular striations were equally present in healthy left ventricular muscle (panel a; Table 1, specimen 2); hypertrophic cardiomyopathic left ventricular outflow tract (panel b, specimen 9); hypertrophic cardiomyopathic left ventricular outflow tract, linked to chromosome 14q1 (panel c, specimen 11; panel d, specimen 12); hypertrophic cardiomyopathic right ventricle β-myosin heavy chain gene 403 Arg to Gln mutation (panel e, specimen 14); and hypertrophic cardiomyopathic left ventricular outflow tract, β-myosin heavy chain gene 403 Arg to Gln mutation (panel f, specimen 13). Bar, 10 μm.

Figure 3. Cardiac β-myosin heavy chain is assembled within hypertrophic cardiomyopathic skeletal myofilaments. Longitudinal section of three adjacent muscle fibers from vastus lateralis muscle of hypertrophic cardiomyopathic patient with β-myosin heavy chain mutation at residue 403 (Arg to Gln) is demonstrated at the DNA level (Table 1, specimens 14 and 17), as viewed by phase-contrast light microscopy (panel A). The fiber at the center stains with anti–myosin heavy chain monoclonal antibody; the surrounding fibers are negative. In the positive fiber, striations observed by immunofluorescence match sarcomeric repeats observed by phase-contrast microscopy (panel B). The small arrows in panels A and B point to A-band striations. The large arrows in panel B point to the boundary of the immunoreactive fiber. Bar, 10 μm.
cardiac muscle.18–27 Accordingly, our results suggest that functional mechanisms rather than disruption of assembly may have to be considered in the development of clinical HCM. Differential functional effects of specific mutant β-MHC alleles may contribute to the broad spectrum of pathology of HCM that extends from asymmetric septal hypertrophy with fiber disarray to symmetrical hypertrophy of the left ventricular free wall and to isolated apical hypertrophy.13–17 Another indication of possible physiological effects of β-MHC mutants stems from the relative lack of pathology in the right ventricle in contrast to the diagnostic hypertrophy of the left ventricle and interventricular septum despite the similarity of their myosin content. The mutation-sensitively left ventricle produces systolic pressure approximately fourfold greater than that of the relatively insensitive right ventricle.

While increased α-MHC/β-MHC ratios may conceivably contribute to the pathogenesis of HCM in some subjects,6 the results of our investigation indicate that this is unlikely to be the case in the subjects studied here. In agreement with a previous report,2 α-MHC is at such low levels in ventricular myocardium in HCM hearts that it is not detected by immunoblotting methods sensitive to subnanogram levels.10 β-MHC remains the predominant ventricular isoform in all samples studied. In skeletal muscle, specific anti–cardiac β-MHC labeled in a regular striated pattern, consistent with the assembly of β-MHC into organized sarcomeres. These results agree with previous reports emphasizing the absence of MHC isoform switching in human ventricles hypertrophied from chronic mitral regurgitation, arterial hypertension, and idiopathic HCM.2,28–30

In summary, myofibrillar organization appears intact in HCM caused by the 403 Arg to Gln β-MHC mutation. Although this study did not distinguish mutant from normal β-MHC molecules in the cardiac biopsies, we hypothesize that subtly defective myofibrils containing the mutant myosin may fail to meet the contractile demands of a developing heart, triggering an abnormal contractile response leading to hypertrophy, the extent and location of which may be allele specific. MHC head mutations in Caenorhabditis elegans that exhibit abnormal contractility in the presence of normal sarcomere structure31,32 provide an experimental model for the dissociation of intact myofibrillar structure and perturbed contractile function observed in human HCM.33

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