Distribution of Cardiac Myosin Isozymes in Cardiomyopathy: 
Immunohistochemical and Gene Analysis

YOSHIKAZU YAZAKI, M.D., HIDETSUGU TSUCHIMOCHI, M.D., MASAHIKO KURABAYASHI, M.D. 
MASATOSHI KAWANA, M.D., AND SHIN-ICHI KIMATA, M.D.

We characterized cardiac myosin isoforms by immunohistochemical approaches using monoclonal antibodies and demonstrated the existence of a distinctive type of cardiac myosin heavy chain which predominates in the fetal stage but is depressed during postnatal development. Furthermore, we showed that this type of cardiac myosin heavy chain was markedly expressed in patients with dilated cardiomyopathy. The results suggested that the pathologic process involved in dilated cardiomyopathy affects myocardial differentiation by the inhibition of the myosin gene switching that normally occurs during muscle maturation. However, we could not find any polymorphism in myosin gene in our population sample of dilated cardiomyopathy. The pathophysiological role of fetal type cardiac myosin expression in dilated cardiomyopathy will be clarified by the characterization of the myosin near future.

MYOSIN is a major contractile protein in muscle cells and has enzymatic activity, ATPase activity, to liberate energy from ATP for muscle contraction. Electrophoretic and immunological studies indicate that multiple myosin isoforms exist among various muscle tissue and that a heterogeneous fiber distribution with respect to myosin composition varies according to the developmental stage or the physiological and pathological stage of the muscle. Each myosin isoform contains a specific combination of heavy and light chains. Recent reports have shown that the fetal myosin heavy chains, disappearing during postnatal development, are transiently expressed in degenerating or regenerating skeletal muscle. Although the physiological role of the appearance of the fetal myosin is still unknown, the myosin molecule appears to be a sensitive marker of the differentiation of muscle fibers. And factors affecting muscle differentiation, and inducing synthesis of different isoforms of myosin could be involved in the pathogenesis of muscle disease.

Since evidence shows that analysis of myosin isoforms can provide insight into pathologic process that may interfere with the normal regeneration of the myocardium, we investigated characterization of cardiac myosin isoforms in patients with cardiomyopathy by immunohistochemical approaches using monoclonal antibodies to clarify the pathogenesis of cardiomyopathy, especially dilated cardiomyopathy of which pathological findings are characterized by degeneration of the myocardium.

In this study, we demonstrated the existence of a distinctive type of cardiac myosin heavy chain which predominates in the fetal stage but is depressed during postnatal development. Fur-
thermore, we found that this type of cardiac myosin heavy chain was markedly expressed in patients with cardiomyopathy.

To search for changes in myosin heavy chain genes in relation to distinctive expression of myosin isoforms in patients with cardiomyopathy, we examined DNA isolated from individual patients by hybridization with the probes.

MATERIALS AND METHODS

Myosins for immunization were prepared from calf fetal ventricular muscles by a modified dilution method. Myosin heavy and light chains were dissociated from the whole myosin molecule by guanidine denaturation.

Immune reaction and preparation of hybridomas were carried out as follows. Briefly, BALB/C mice were immunized by five intraperitoneal injections at two week intervals with 50–100 μg of myosin emulsified in Freund's adjuvant. Ten weeks after the initial injection, the animals were given an intravenous boost injection of 30 μg of antigen. Animals exhibiting high serum titer were killed 5 days after the intravenous injection, and their isolated spleen cells were fused with cells of the myeloma line P3 × 63Ag8Ul according to the Kohler and Milstein procedure. After recovery in HAT selective medium, the hybrid cells were cultured in dishes for 7 days. Thereafter, the hybridoma colonies were screened for specific antmyosin antibodies by enzyme-linked immuno-sorbent assay (ELISA).

The distribution of myosin isoforms in human heart muscle was investigated by immunofluorescence assay. Human myocardial specimens were obtained from patients without heart disease or with dilated cardiomyopathy at autopsy or biopsy. The tissues were washed with saline and frozen sections were stained with the indirect immunofluorescence procedure as follows. The sections were incubated for 30 min at 37°C with unlabeled monoclonal antibodies in appropriate dilutions, final antibody concentrations being about 0.2 μg/ml. Then the sections were washed with phosphate-buffer saline and treated with biotinylated goat antimouse immunoglobulin and FITC-labeled avidin for 40 min at 37°C.

Six patients with familial dilated cardiomyopathy were selected from the cardiology clinics at Kurume University. Control samples were taken from healthy volunteers. DNA was extracted from whole blood by Bell's method. Restriction endonuclease digestions were carried out according to the manufacturer's instructions. DNA was digested with the following restriction

![Image](https://example.com/image)

Fig.1 (A) Cryostat section of human embryonic heart stained with CMA19. All myofibers reacted strongly in the atrium (At), but were not stained in the ventricle (V). (B) As in A, except that the section was stained with HMC14. All myofibers in the ventricle are strongly labeled. Note that the myofibers in the atrium were not stained. (C) As in A, except that the myofibers were stained with HAL8. All myofibers in both atrium and ventricle reacted strongly and homogeneously.

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enzymes BamHI, SstI, TaqI and MspI (under manufacturer's conditions Takara Shuzou). DNA fragments were separated by size on a 0.8% agarose gel and were transferred to 'Hybond' filter (Ammersham) by the Southern blotting technique. The mouse cardiac myosin heavy chain probe pMHC101 was a kind gift from Dr. M.E. Buckingham and encodes the 3' end of the MHC cDNA clone. The probe was labeled by means of nick translation to a specific activity of \(2 \times 10^8\) cpm/\(\mu\)g. Each filter was hybridized with \(1 \times 10^7\) cpm at 42°C for 18h and then washed finally in 1 x SSC (0.15 mon/l NaCl; 0.015 mol/l sodium citrate), 0.1% SDS. The filters were exposed to X-ray film for 2 days.

RESULTS

Histochemical study using monoclonal antibodies

We developed three monoclonal antibodies specific for human cardiac myosin heavy chain \(\alpha\) and \(\beta\) type, and atrial light chain (CMA19, HMC14 and HAL8, respectively). In this study, we raised the monoclonal antibody specific for fetal cardiac myosin heavy chain (FCH21) selected by ELISA. This was confirmed by immunohistochemical studies as follows.

1) Fetal heart

Distribution of myosin isoforms was indicated in the fetal atrium and ventricle by indirect immunofluorescence staining. In the atrium, all myofibers were reactive with CMA19 strongly and homogeneously, but any myofiber was not stained with HMC14. In contrast, all ventricular myofibers were reactive with HMC14, and a small number of them were also stained with CMA19. Since they were distributed in the endocardial region they seemed to be Purkinje fibers. FCH21 stained ventricular myofibers strongly. Interestingly, HAL8 reactive with the atrial alkaline light chain stained all myofibers of the fetal ventricle homogeneously as well as those of the atrium. No difference in the reactivity with the antibody was observed between them (Fig. 1).

2) Normal adult heart

A comparison of the staining patterns of myofibers with individual monoclonal antibodies between adult and fetal hearts revealed some redistributions of myosin isoforms occurring during developmental stage. In the atrial myocardium, the myofibers reactive with HMC14 were increased and comprised from 20 to 40% of total myofibers. They were mainly interspersed and could not be distinguished from unreactive myofibers by size or morphological criteria. Again, the myofibers reactive with CMA19 appeared among ventricular working myocardium. However, their proportion showed significant regional variation. The number of labeled myofibers increased gradually from the subendocardial to the subepicardial region, where up to 15% of the myofibers were stained by CMA19 (Fig. 2).

Interestingly, the number of fibers reactive with FCH21 decreased in the adult ventricular myocardium to 40% of total myofibers, whereas all myofibers were stained homogeneously in the fetal ventricle (Fig. 3). Furthermore, the number of reactive myofibers with HAL8 were markedly decreased in the adult ventricular myocardium, forming a striking difference in reactivity from

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Fig.2. Ventricular myocardium stained with CMA19. (A) A section from the subepicardial region. A greater number of myofibers is labeled than in panel B. (B) A section from the subendocardial region. Almost all myofibers are unlabeled.

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the atrium where all myofibers were still strongly stained. The reactive myofibers of the ventricle were mainly observed in the conduction system.

3) Dilated cardiomyopathy

Staining patterns of the myofibers with CMA19 and HMC14 observed in patients with dilated cardiomyopathy were not significantly different from those of normal adult heart. Almost all of atrial and ventricular myofibers were stained with CMA19 and HMC14, respectively. The number of reactive myofibers with CMA19 was not increased in the ventricle compared with that of normal adult ventricle. However, a striking difference was found in the reactivity of ventricular myofibers with FCH21 between normal and cardiomyopathy. Almost all myofibers were reactive with the antibody in the ventricle of patients with cardiomyopathy, whereas less than a half of myofibers were unreactive in the normal ventricle (Fig. 4). On the other hand, we failed to find any difference in the staining patterns of myofibers with HAL8 between normal and cardiomyopathy. The reactive myofibers were also distributed in the subendocardial region of the ventricle, corresponding to the conduction system, in patients with cardiomyopathy.

Analysis of cardiac myosin heavy chain gene

To study the population polymorphism in myosin heavy chain genes, we examined DNA isolated from different individuals for changes in myosin heavy chain gene sequences that would result in alteration of restriction fragment patterns. We investigated the hybridization spectrum to DNA isolated from 6 patients with DCM and the healthy individuals that we treated.

The pattern of hybridizing bands was identical for each of the 13 (DCM 6, Normal 7) DNA examined. Similarly when DNA from these 13 individuals was cleaved with the restriction enzymes MspI or TaqI and hybridized to the same probes, no differences in the hybridization banding patterns were observed between the individual’s DNA for either of the enzymes utilized. Thus, at the level of restriction enzymes site and fragment length changes, we were unable to detect any polymorphism in myosin gene-containing fragments in this small population sample.

DISCUSSION

In this study, we demonstrated the presence of a distinctive cardiac myosin heavy chain, which was predominantly expressed in the fetal ventricle but depressed during postnatal development, by immunohistochemical study using monoclonal antibodies. Furthermore, we revealed that this type of cardiac myosin heavy chain was markedly expressed in patients with cardiomyopathy. However, in the case of myosin light chain, atrial light chain which resembles fetal myosin light chain, was not significantly increased in the ventricular myocardium of the patients.

Recently, monoclonal antibodies which react with specific antigenic determinants on the protein molecule have been developed for the discrimination of a specific protein among structurally similar molecules. We have prepared two types of monoclonal antibodies, CMA19 and HMC14 specific for human α and β type of
myosin heavy chains (HCα and HCβ), respectively. By immunofluorescence assay, we demonstrated that although HCα and HCβ are predominant isoforms in the atrium and ventricle, respectively, HCα and HCβ also coexist in both the atrium and ventricle. Furthermore, we demonstrated that in pressure overloaded atria, the HCβ content increased strikingly while that of HCα showed a corresponding decrease. This atrial HCβ was also found to be essentially the same HCβ in the ventricle in its peptide composition and enzymatic activities.

In this study, we examined the expression of these myosin isoforms from fetal through adult cardiac tissue immunohistochemically. From early developmental stage, HCα and HCβ were expressed in the atrium and ventricle, respectively. HCβ was expressed in the atrium after birth and increased up to 40% of total myosin heavy chain. HCα was expressed even in the fetal ventricle, but localized in the conduction system. After birth, ventricular HCα was mainly observed in the epicardial region.

We also examined the distribution of the myofibers reactive with FCH21, which was a newly raised monoclonal antibody by using calf fetal myosin heavy chain as an antigen. By immunohistochemical assay using FCH21, we revealed the presence of the distinctive isoform of cardiac myosin heavy chain (HCf). HCf was predominantly expressed in the fetal ventricle but repressed in the adult. However, in cases of dilated cardiomyopathy, HCf was markedly expressed as observed in the fetal ventricle. Evidence shows that the fetal type of skeletal myosin, disappearing during postnatal development, is transiently expressed in degenerated skeletal muscle disease. This observation suggests that the pathologic process involving in dilated cardiomyopathy affected myocardial differentiation. However, the pathophysiological role of HCf expression in cardiomyopathy was not elucidated since characteristics of HCf have not been determined completely. All ventricular myofibers reactive with FCH21 were also stained with HMC14. Correspondingly, HCf appeared to be a subtype of HCβ. Furthermore, the expression of HCf was not increased in the overloaded myocardium (data not shown).

Recently, Hirzel et al. reported that the content of atrial myosin light chain (ALC) resembling fetal light chain was significantly increased in cases of dilated cardiomyopathy. To confirm their observation, we examined the distribution of ALC by immunohistochemical analysis using a monoclonal antibody, HAL8, specific for atrial light chain. The proportion of the ventricular myofibers expressing ALC was relatively small, but showed significant regional variation. In contrast to HCα, the relatively high ALC content was observed in the myofibers of subendocardial region. However, we observed the wide variations among individuals, and could not find statistical difference between the cardiomyopathy group and the controls. Even though the expression of ALC in the ventricular tissue is related in some way to dilated cardiomyopathy, one would expect that the increment of ALC does not play an important role in contractile function, since the heavy chain largely determines myosin ATPase activity which controls muscle contraction biochemically.

These studies suggested that the pathological process involving in cardiomyopathy affects myocardial differentiation by the inhibition of the myosin gene switching that normally occurs during muscle maturation.

To study the genetic alteration of cardiac myosin, we analyzed polymorphism in myosin heavy chain genes. However, at the level of restriction enzymes site and fragment length changes, we could not find any polymorphism in myosin gene in our small population sample of dilated cardiomyopathy. Further analysis of myosin isoforms and genes could provide insight to pathologic process involving in dilated cardiomyopathy.

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