Heterogeneity of β-Type Myosin Isozymes in the Human Heart and Regulational Mechanisms in Their Expression

Immunohistochemical Study Using Monoclonal Antibodies

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Abstract

To investigate the existence of heterogeneity of β-type myosin isozymes (HCβ) in human hearts, immunohistochemical studies using monoclonal antibodies (MoAbs) raised against human ventricular myosin heavy chains were performed. Two types of MoAbs recognized some muscle fibers in the atrium, whereas both reacted with all ventricular muscle fibers. Since atrial muscle fibers reactive with each MoAb were found to be clearly different, the existence of two immunologically distinct HCβ (β_1, and β_2) was suggested in the atrium. By using affinity chromatography, two molecular variants of HCβ were isolated from the bovine atrium, and differences in the primary structure of β_1 and β_2 were confirmed by analysis of peptides produced by chymotriptic digestion. In pressure-overloaded human atria, myofibers containing β_1 and/or β_2 increased in accordance with decrement of myofibers containing α-type myosin isozyme ($P < 0.01$). But they differed in expression during the developmental stage, since β_2 did not exist in the early embryonic bovine heart, but β_1 did. Thus, there are two distinct HCβ whose expression is regulated by at least two factors: pressure overload and developmental stage.

Introduction

Myosin, a major contractile protein in muscle, is composed of two 200,000 D heavy chains (HCs)[1] and two pairs of low molecular weight light chains (LCs) (1). HC largely determines myosin ATPase activity (2), which is known to be correlated with the contractile velocity of the muscle (3–5), and hence, it is an important subunit in contractile function. In the cardiac muscle, two types of HCs, HCα and HCβ, encoded by two different genes (6), which differ in ATPase activity (HCα has a higher Ca^{2+}- and actin-activated ATPase activity than does HCβ) were found in the ventricular myocardium of laboratory animals such as rats, mice, and rabbits (7–9). Until recently, owing to the difficulty in separating isozymes with polyacrylamide gel electrophoresis, the existence of these isozymes in human and bovine hearts had not been clarified. But in these past few years, immunological studies from our laboratory (10, 11) and others (12, 13) clearly demonstrated the presence of HCα and HCβ in human ventricles, where HCβ was a predominant myosin isozyme. More recently, the presence of a third type of myosin isozyme, which belonged to HCβ, was suggested by the amino acid sequence of bovine ventricular myosin subfragment-1 (14), and by immunohistochemical studies in the human heart (15). However, definitive evidence supporting the existence of the heterogeneity of HCβ and regulational mechanisms in their expression have not been obtained yet.

In this study, we prepared MoAbs raised against human ventricular myosins in order to clarify the existence of the heterogeneity of HCβ and to demonstrate their distribution. By immunofluorescence studies and peptide analysis of each affinity purified myosin, we found the presence of two subtypes of HCβ and, for the first time, showed the distribution of these isozymes in human hearts. In addition, we examined the expression in pressure-overloaded atria and fetal hearts. Our results show that the expression of these isozymes is regulated by at least two factors: pressure overload and developmental stage.

Methods

Production of MoAbs. Myosins were isolated from bovine atria and human ventricles by a dilution technique (16). The LCs (I, II) were isolated from human ventricles by guanidine denaturation, described elsewhere (17). The HC and LCs used in the inhibition study were purified by electroelution from the SDS-PAGE as described by Umeda et al. (18). MoAbs specific for HCα and HCβ were produced by fusion of isolated spleen cells from mice immunized by myosins purified from bovine atria and human ventricles, respectively, with myeloma cell lines (P_3U_1) (10). Antimyosin activity in the medium from hybridoma colonies was screened by ELISA according to Guesdon et al. (19), as described previously (10, 11).

Immunoblotting. The specificity of antimyosin MoAbs was further clarified by Western immunoblots according to Towbin et al. (20). In brief, human ventricular or bovine atrial myosin was subjected to SDS-PAGE (%T = 12.5%, *T:* the percentage concentration of acrylamide + methylenebisacrylamide) performed as described by Laemmli (21), then electrophoretically transferred to nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) at 6 V/cm for overnight at 4°C in 25 mM Tris HCl, 192 mM glycine, 20% methanol. After washing with 10 mM PBS (pH 7.4) with 0.05% Tween 20, the membrane was cut out and one was stained with amido black, while the others

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1. *Abbreviations used in this paper:* ALCI, atrial light chain I; AR, aortic regurgitation; AS, aortic stenosis; ASR, aortic stenosis and regurgitation; HC, heavy chain; LC, light chain; MR, mitral regurgitation; MS, mitral stenosis; MSR, mitral stenosis and regurgitation; TRITC, tetraethyl rhodamine isothiocyanate; TR, tricuspid regurgitation; VLCI, ventricular light chain I.

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were reacted with MoAbs. Bound antibodies were detected with horseradish peroxidase-conjugated goat antibody to mouse IgG and color development reagent containing 4 chloronaphthol as described in the protocol of the immune blot assay kit (Bio-Rad Laboratories).

Inhibition study. Inhibition study by the ELISA method was performed as follows: after HC or LCs (I, II) purified by electroelution were dialyzed to 1 mM acetate buffer (pH 4.4) overnight, followed by dialysis with 100 mM PBS (pH 7.4), serial dilutions of each antigen were incubated with a MoAb at 4°C overnight. Aliquots were applied to wells of 96-well microtiter dishes coated with native human ventricular myosin. After washing with PBS, bound antibodies were detected by ELISA.

Immunohistochemical studies. Immunohistochemical studies were performed as described previously (10, 11). To examine the same field stained by various MoAbs, a cryostat section was stained with one MoAb and the other of a serial one was double-stained with two MoAbs with FITC- or tetraethyl rhodamine isothiocyanate(TRITC)-conjugated rabbit antimouse IgG subclass (Nordic Immunological Laboratories, Tilburg, The Netherlands). For example, since the subclasses of HMC14 and HMC50 were IgG_{2a} and IgG_{2b}, respectively, a cryostat section was first incubated with HMC50 and then treated with TRITC-labeled antimouse IgG_{2b}; after washing with PBS, the same section was further incubated with HMC14 and finally treated with FITC-labeled antimouse IgG_{2a}. These sections were examined under a microscope equipped with fluorescence optics (Nikon, Tokyo, Japan) with filters for FITC or TRITC. The staining intensities of myofibers with a MoAb were divided into four classes: "strongly positive" (S), "positive" (P), "pseudonegative", and "completely negative". We calculated the percentage of myofibers reacted with a MoAb as P (%) + S (%) by counting more than 1,000 myofibers. As for quantification, total scores were calculated, scoring one S fiber as one, one P fiber as 0.5, and one pseudonegative or completely negative fiber as 0, as described previously (10). Thus, the percentage of myofibers reactive with a MoAb and the total scores per 1,000 myofibers were calculated in each specimen.

Preparation of affinity columns and purification of each myosin isozyme. Ascites fluids containing antimyosin antibodies were produced in Balb/c mice by intraperitoneal injection of hybridoma cells following injection of 2,6,10,14-tetra methyl penta-decane (Sigma Chemical Co., St. Louis, MO). Each IgG was purified by affinity columns using protein A Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) or a monoclonal antibody purification system kit (Bio-Rad Laboratories) according to the manufacturer's protocol. After dialysis against coupling buffer (0.1 M $NaHCO_3$, pH 8.0, containing 0.5 M NaCl), each IgG was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) in a ratio of 5 mg of IgG to 1.0 ml of activated gel, which was previously washed with 200 ml of 1 mM HCl. The reaction proceeded for 2 h at room temperature or 16 h at 4°C, then, after removal of the protein solution, an equal volume of 0.2 M glycine (pH 8.0) was added and the reaction was allowed to proceed for an additional 2 h at room temperature. The gels were exhaustively washed with alternating high (coupling buffer) and low (0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl) buffers and then with a column buffer (20 mM Tris, 0.5 M KCl, pH 7.5).

Myosin was applied to the column at 4°C overnight. The unbound protein was washed with the column buffer until the absorbance at 280 nm of the effluent was 0.01 or less. The bound fraction was eluted with 4 M guanidine-HCl in the column buffer (22), and immediately dialyzed against the column buffer. To eliminate contamination by heterodimer, the purified myosin from one column was exhaustively absorbed by the other two columns, and the unbound myosin fraction was collected and concentrated to ~ 1 mg/ml using an ultrafiltration kit (Immersible CX-10; Millipore Corp., Bedford, MA).

Chymotriptic digestion of myosin and one-dimensional gel electrophoresis of peptide fragments. The partial proteolytic cleavage of myosin with chymotrypsin was performed according to the method of Cleaveland et al. (23). Briefly, myosin (0.5 mg/ml) was cleaved with α-chymotrypsin (Worthington Biochemicals, Freehold, NJ) in the presence of SDS, at a myosin to chymotrypsin ratio of 8:1, for 30 min at 37°C. The reaction was terminated by boiling for 3 min in the presence of 3% SDS and 6% 2-mercaptoethanol. Peptide fragment were separated by electrophoresis in the presence of SDS on 13% acrylamide slab gels using the Laemmli buffer system (21). Digests of myosin (~ 10 μg) were applied to each gel slot, and electrophoresis was carried out at a 15-mA constant current for 14 h at room temperature. Gels were stained with a silver staining kit (Ag-Stain, Daiichi; Daiichi Pure Chemicals, Tokyo) according to the manufacturer's protocol.

Specimens. Human cardiac tissues were obtained at cardiac surgery from patients with acquired valvular disease or ischemic heart disease (n = 23; age: 19–74 yr; mean±SD: 52.1±10.6 yr). The correct clinical diagnoses of these patients were ischemic heart disease (n = 5), mitral stenosis (MS; n = 4), mitral regurgitation (MR; n = 5), MS and MR (MSR; n = 2), aortic regurgitation (AR; n = 1), aortic stenosis (AS) and AR (ASR; n = 1), MR and AR (n = 1), MS and AR (n = 1), and MSR and ASR (n = 3). In addition to these primary lesions in mitral or aortic valves, some patients had secondary tricuspid regurgitation (TR), which was detected by doppler echocardiography or cardiac catheterization or observed at surgery. Cardiac catheterization was performed in all patients within four weeks before cardiac surgery, and the right atrial and pulmonary capillary wedge pressure were recorded in each patient. Fetal bovine cardiac tissues were obtained from animals after ~ 2–8 mo of gestation.

Other methods. The concentration of protein was measured as described by Lowry et al. (24). Linear regression analysis was carried out by the conventional method of least squares.

Results

Characterization of MoAbs. Three MoAbs specific for human myosin isozymes were prepared and used in this study. Of these MoAbs, one (CMA19) was specific for HCα, and the others (HMC14 and 50) were specific for HCβ. Fig. 1 demonstrates their specificities to each isozyme by the ELISA tests (A), immunoblotting (B), and inhibition study by the ELISA method (C). In the ELISA tests, CMA19 reacted with atrial myosin specifically, and HMC14 and 50 reacted selectively with the ventricular myosin (10, 11). Neither of these MoAbs reacted with LCs (I, II) (Fig. 1 *A*). The slight reactions of CMA19 with ventricular myosin and those of HMC14 and 50 with atrial myosin were due to the coexistence of atrial-type myosin in the ventricle and ventricular-type myosin in the atrium, respectively. This was confirmed by immunohistochemical studies. In immunoblot analyses with these MoAbs, HMC50 reacted strongly with human ventricular HC, but CMA19 reacted weakly. HMC14 did not react with antigens on nitrocellulose membrane. When atrial myosin was examined by this method, CMA19 reacted strongly with its HC as did HMC50, whereas HMC14 did not react with this antigen (Fig. 1 *B*). Since HMC14 immunoprecipitates HC, LC I, and II (when reacted with native ventricular myosin in 20 mM Tris, pH 7.5, 0.5 M KCl, and subjected to SDS-PAGE; see Fig. 4), an inhibition study was undertaken. When purified HC was treated with 1 mM acetate buffer, followed by dialysis with 100 mM PBS, the HC clearly inhibited the reaction of HMC14 with native ventricular myosin. However, the LCs (I, II) obtained by the same method did not (Fig. 1 *C*). Therefore, HMC14 appeared to react with a determinant of the HC, which was easily denatured by various factors including SDS, but recovered by dialysis with acetate buffer. Thus, all these MoAbs reacted with HCs. Since a previous report from our laboratory demonstrated that atrial myosin had a higher ATPase activity than did ventricular myosin in the human heart

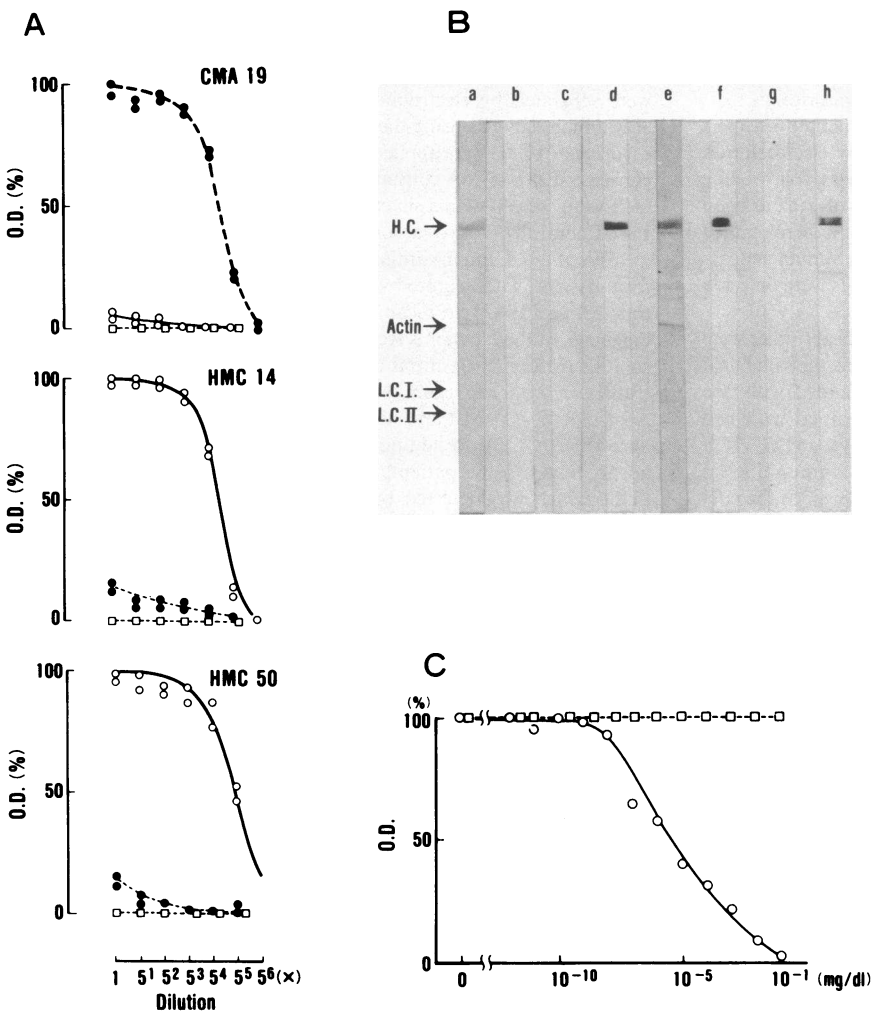

Figure 1. Analysis of MoAbs by ELISA tests (*A*), Western immunoblots (*B*), and inhibition studies of HMC14 by the method of ELISA (*C*). (*A*) CMA19 reacted with atrial myosin selectively and reacted negligibly with ventricular myosin. In contrast, HMC14 and 50 reacted specifically with ventricular myosin and also showed a slightly positive reaction with atrial myosin. Neither of these antibodies reacted with LCs (I, II). ●, bovine atrial myosin; ○ human ventricular myosin; □, LCs (I, II) purified from human ventricles. (*B*) When ventricular myosin resolved by SDS-PAGE was transferred to nitrocellulose membrane (*a–d; a* amido black stain), HMC50 reacted strongly with 200,000-D HC (*d*), but HMC14 did not react with the antigens on nitrocellulose membrane (*c*). CMA19 reacted weakly with the HC (*b*). When atrial myosin was similarly used (*e–h; e,* amido black stain), CMA19 reacted strongly with the HC (*f*) but HMC14 did not react (*g*). HMC50 also reacted with the HC of atrial myosin (*h*). To obtain sufficient reaction with each MoAb, each membrane was incubated in the reaction buffer for as long as required. HMC14 did not react with any of the protein on nitrocellulose membrane even if reacted for a longer time. (*C*) HMC14 was shown to be specific for the HC, because purified ventricular HC (○), but not LCs (I, II; □) after treatment with 1 mM acetate buffer, clearly inhibited the reaction of HMC14 with native human ventricular myosins. (See text for details.)

(25), and we know that HCα has a higher ATPase activity than does HCβ, HCs reactive with CMA19 and HMC14 or HMC50 were regarded as HCα and HCβ, respectively. These conclusions were further confirmed by immunohistochemical studies.

Distribution of each myosin isozyme in normal human hearts. Fig. 2 shows the human ventricle and atrium stained by these MoAbs. Although HMC14 and 50 stained all ventricular myofibers, CMA19 stained only a small number of them (Fig. 2 *A–C*). In contrast, almost all human atrial myofibers were stained by CMA19, whereas some myofibers were also stained by HMC14 and HMC50 (Fig. 2 *D–F*). Serial sections stained by HCβ-specific MoAbs revealed that the staining pattern of HMC50 was clearly different from that of HMC14 (Fig. 2 *E, F*). The number of muscle fibers stained by HMC50 was roughly two to three times more than those stained by HMC14. These results suggested the presence of two immunologically distinct β-type HCs in the human atrium. Further clarification of their distribution in the human atrium was obtained by examining the same field of serial sections stained by CMA19 and double-stained by HMC14 and HMC50 (Fig. 3). We designated HCβ detected by HMC14 as β_1 and that by HMC50 as β_2 (see Discussion for nomenclature). Although almost all human atrial myofibers contained HCα dominantly (*a*), some myofibers contained also β_1 and/or β_2 (*b, c, g*). In addition, there were myofibers that dominantly expressed β_1 (*f*), or β_2 (*d*), or both (*e*), without expression of HCα. Therefore, atrial myofibers expressed two immunologically distinct HCβ and HCα in various combinations. Thus, at least three immunologically distinct myosin isozymes were suggested to exist in the human atrium.

Separation and identification of three molecular variants of myosin heavy chain from the bovine atrium. In the immunohistochemical study of the bovine heart, these MoAbs stained the atrium and ventricle in almost the same pattern as they did in the human heart. Therefore, it was suggested that there were also three myosin isozymes in the bovine atrium. To determine whether there existed a difference in the primary structure of HCα, β_1, and β_2, we isolated each myosin from the bovine atrium by affinity chromatography, and evaluated each by comparison of the fragments produced by partial proteolytic digestion of myosins. Without proteolytic digestion, each affinity purified myosin was very pure, containing HC and LCs I and II (Fig. 4). The band of LC II was very faint, probably because LC II tends to be lost during the dilution procedure especially when myosin is extracted from the atrium. All accompanying LCs had the same mobility in SDS-PAGE as atrial LCs, and therefore it was concluded that β_1 and β_2 in the atrium accompanied atrial LCs but not ventricular type LCs. Thus, since all three fractionated myosins had the same LCs,

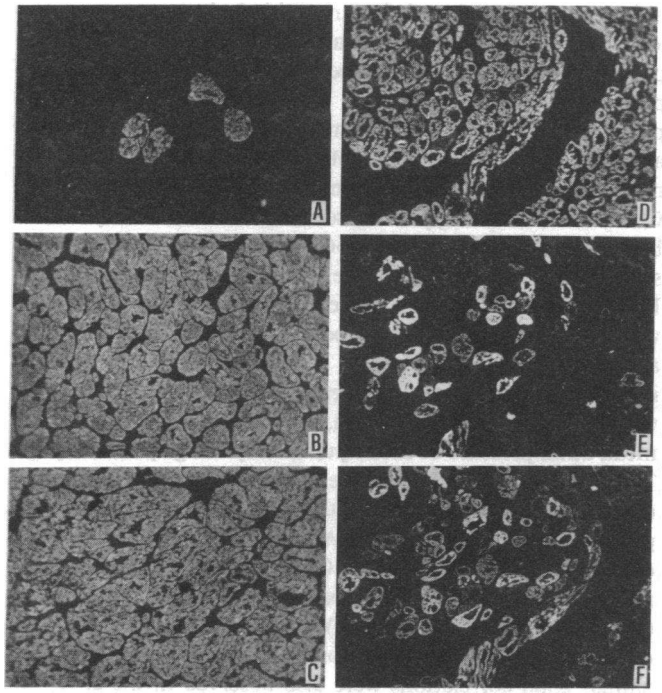

Figure 2. Analysis of MoAbs by immunohistochemical studies. *A–C* ventricle; *D–F*, atrium; stained by CMA19 (*A, D*), HMC14 (*B, E*), and HMC50 (*C, F*). CMA19 (*A*) stained a few myofibers but HMC14 (*B*) and 50 (*C*) stained all myofibers of the human ventricle. Although CMA19 (*D*) stained almost all atrial myofibers, HMC14 (*E*) and HMC50 (*F*) stained only some myofibers. In serial atrial sections, the staining pattern of HMC14 was found to be clearly different from that of HMC50 (*E, F*).

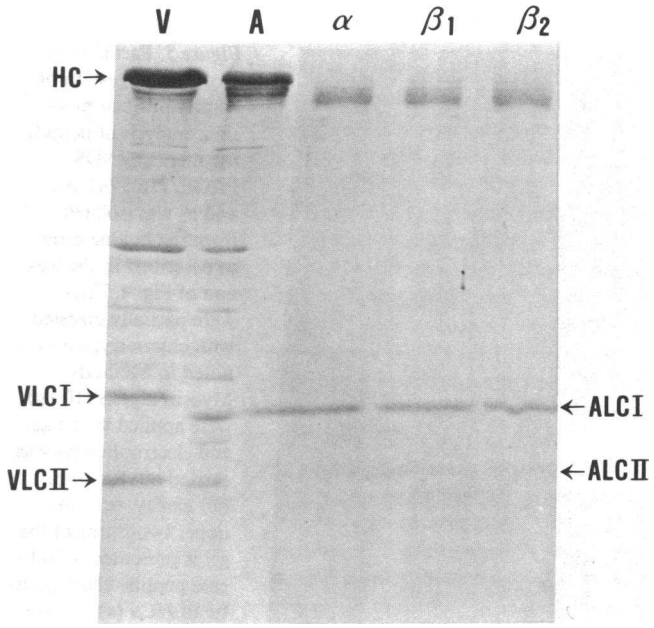

Figure 4. Affinity purified myosin isozymes subjected to SDS-PAGE. HCα(α), β_1, and β_2 were isolated from the bovine atrium by immunoadsorption on affinity columns, as described in Methods. After dialysis and concentration, samples were analyzed by SDS-PAGE. Each isomyosin was very pure compared with ventricular (*V*) and atrial (*A*) myosins isolated by a dilution technique and consisted of HC and atrial LCs (*I, II*). Atrial LC II was very faint because of its loss during the procedure.

as revealed by SDS-PAGE, HC was again suggested to be a target epitope of HMC14. To further determine the difference in the primary structure of each HC, we performed partial tryptic digestion of each myosin by α-chymotrypsin. Fig. 5 shows the peptide fragments of HCα, β_1, and β_2 analyzed by one-dimensional SDS-PAGE. For clarity, only the upper two-thirds of the gel that contained peptide fragments higher than LC I in molecular weight was demonstrated. Although digests of each myosin were similar, each had several specific peptide fragments that were not or hardly detected in the digests of other HCs. In the comparison of HCα and β_1, although they showed a very similar peptide map pattern, there were some

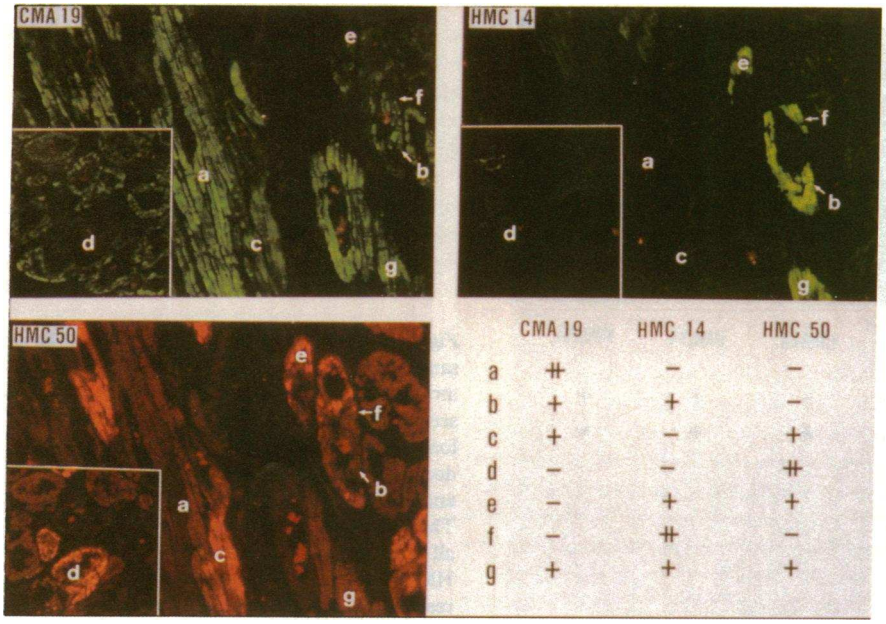

Figure 3. Distribution of three isozymes in the same field of the normal human atrium. CMA19 was treated with FITC-labeled antimouse IgG. HMC14 and HMC50 were treated with FITC-labeled antimouse IgG_{2a} and TRITC-labeled antimouse IgG_{2b}, respectively. In the table presented in the lower right panel, typical myofibers labeled with each MoAb are summarized.

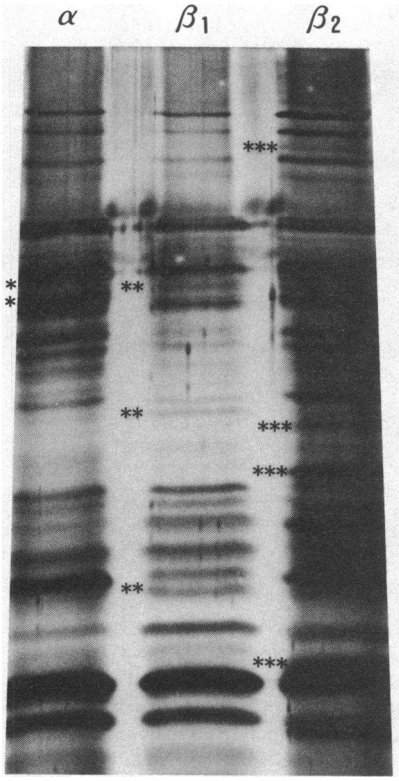

Figure 5. Partial chymotryptic digestion of affinity purified myosins: analysis of peptide fragments by SDS-PAGE. *HCα (α), β_1,* and β_2 was isolated from the bovine atrial as described in the legend of Fig. 4. They were partially digested with chymotrypsin as noted in Methods. Myosin digests (10 μg) were applied to the gel and electrophoresis was carried out for 14 h. For clarity, only the upper two-thirds of the gel is presented. * Indicate peptide characteristic to *HCα* (*), β_1 (**), and β_2 (***). The peptide fragments of β_1 (**) were not specific to only β_1, but were hardly detected in HCα and β_2.

minor bands (*, **) present in one but hardly detected in the other. β_2 also differed from HCα and β_1 in the presence of some major bands (***) that were not detected in HCα and β_1. Therefore, it was concluded that HCα, β_1, and β_2 were distinct molecules. Thus, it was confirmed that at least three myosin HCs that were different in the primary structure from each other were expressed in the atrium.

Redistribution in pressure-overloaded atria. We reported the isozymic redistribution in human atria by pressure overload, that is, HCα, a predominant myosin isozyme in the atrium was replaced by β_1 in atria subjected to pressure overload. The extent of isozymic change correlated well with atrial pressure (10). Here, we observed a similar increment of β_2 in pressure-overloaded atria. Fig. 6 shows the same field of a pressure-overloaded right atrium stained by each MoAb from a patient who suffered from MR and TR and whose mean right atrial pressure was 19 mmHg. Myofibers reactive with CMA19 were strikingly decreased, while almost all myofibers became to reactive with HMC50 and HMC14. Thus, in pressure-overloaded atria, myofibers containing predominantly HCα (*a* in Fig. 3) were decreased or were not observed, whereas myofibers containing both β_1 and β_2 with (*g* in Figs. 3 and 6) or without (*e* in Figs. 3 and 6) expression of HCα increased. We calculated the percentage of myofibers reactive with HMC50 in each specimen, and plotted them against the mean atrial pressure or mean pulmonary wedge pressure (Fig. 7 *A*). It was clear that the degree of the increment of myofibers reactive with HMC50 correlated well with the mean atrial pressure ($r = 0.69$, $P < 0.001$). Also, by calculating total scores of β_2 as described previously (10), we found again a good correlation between the total scores of β_2 and mean atrial pressure (Fig. 7 *B*, $r = 0.77$, $P < 0.001$). Furthermore, when separate calculations were carried out for groups of right and left atria, similar good correlations were also observed in the group of right atria (myofibers reactive with HMC50; $r = 0.70$; $P < 0.01$; total scores of β_2; $r = 0.80$, $P < 0.001$), but the group of left atria did not show such correlations (even in calculation of total scores). This was because almost all left atria used in this study had been subjected to pressure overload due to mitral valve disease, and hence, almost all fibers reacted with HMC50 in such specimens. Thus, β_2 also increased according to pressure overload, as did β_1.

Redistribution during the developmental stage. We found that the expression of β_2 was developmentally regulated. As shown in Fig. 8, although HMC14 stained the fetal bovine left ventricles from the early embryonic stage through the adult stage, HMC50 did not react with ventricular myofibers after 2 and 5 mo of gestation (2 mo, 5 mo), but showed a heterogeneous staining pattern with those of 8 mo and reacted strongly

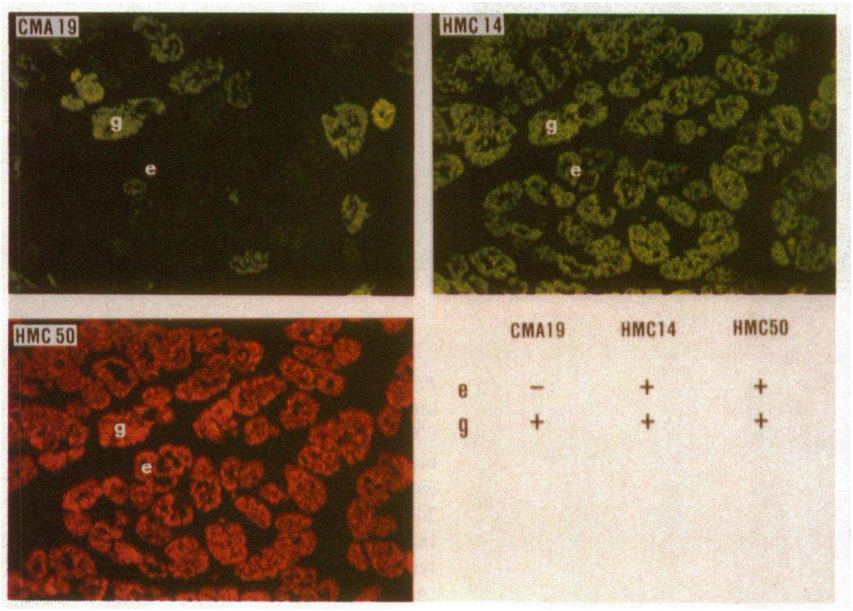

Figure 6. Distribution of three isoforms in the same field of the pressure-overloaded right atrium. Double-staining was performed as described in legend in Fig. 3. In the pressure-overloaded atrium, myofibers reactive with CMA19 decreased, while those reactive with HMC14 and HMC50 showed a corresponding increase. Thus, as shown in the right lower panel, almost all myofibers reacted only with HMC14 and HMC50 (e), but few myofibers still remained to react with CMA19 (g).

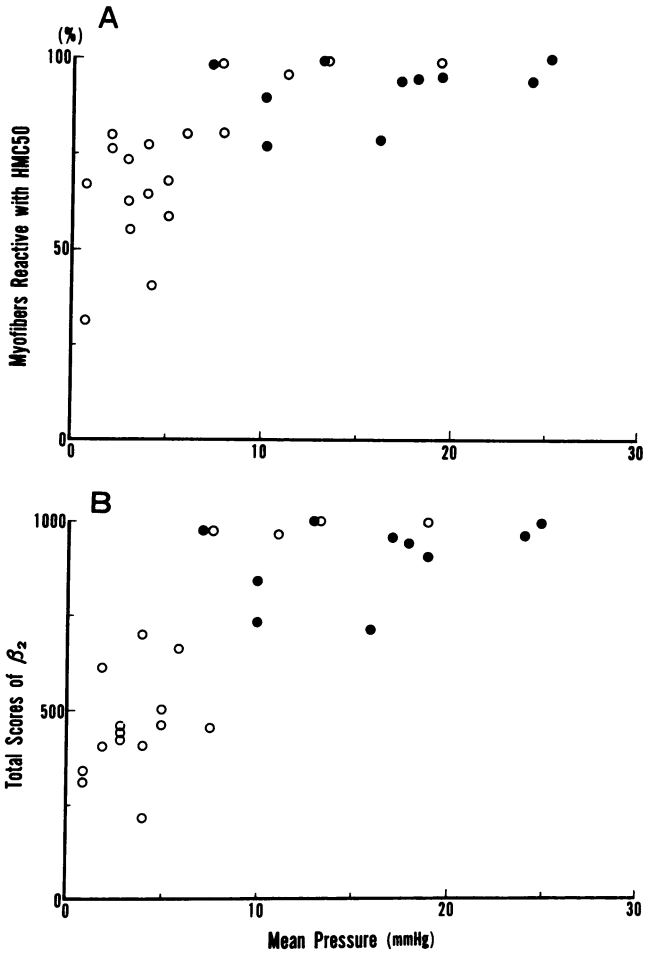

Figure 7. Correlations between mean pressure and the expression of β_2 in human atria. The myofibers reactive with HMC50 (*A*), and the total scores of β_2 (*B*) were plotted against the mean atrial pressure. The myofibers reactive with HMC50 increased according to pressure-overload ($r = 0.69$, $P < 0.001$). The total scores of β_2 also correlated well with the mean atrial pressure ($r = 0.77$, $P < 0.001$). ○, right atrium; ●, left atrium.

with adult ventricular myofibers as did HMC14. CMA19 hardly stained early and late embryonic ventricular myofibers. The same staining pattern during the developmental stage was observed in other ventricular sites such as the interventricular septum and right ventricular free wall. In the atrium, CMA19 stained all myofibers and HMC14 stained some myofibers from the early embryonic stage. However, HMC50 stained some myofibers only after the late embryonic stage when it began to react with ventricular myofibers. Therefore, it was suggested that β_2 was not present in the early embryonic stage, whereas β_1 was expressed from the early embryonic stage through the adult stage. Thus, HCβ expressed in fetal hearts was different from HCβ present in adult hearts in the composition of HCβ subtypes.

Redistributions observed are not phenomena produced by alteration in the binding of MoAbs due to LCI isoform transition. Although these MoAbs were shown to detect epitopes present in the HCs, the binding of MoAbs to these epitopes may be altered by the changes of molecules that associate with HCs. Of these molecules, LCI is a possible candidate, since it has been reported to undergo isoformic transition during development and in response to pressure-overload (26, 27). To eliminate this possibility, we examined the distribution of LCI isoforms in the human atrium and ventricle. We developed MoAbs, HAL8 (IgG_1) and HME17 (IgG_1), specific for atrial and ventricular LCI (ALCI, VLCI), respectively (Tsuchimochi, H., et al., manuscript in preparation). As shown in Fig. 9, the same field of ventricular myocardium was double-stained by HME17 (A) and HMC50 (B). All myofibers were homogeneously reacted with both MoAbs. Since all ventricular myofibers were also double-stained homogeneously by HMC14 and HME17 (data not shown), the presence of VLCI might not disturb the binding of HMC14 and 50 to HCs in the ventricle. Furthermore, atrial tissues were examined by the same method (*C–F*). HME17 stained atrial myofibers that were stained (*a* in *C* and *D*) or not stained (*b* in *C* and *D*) by HMC14. In addition, there existed some myofibers that reacted only with HMC14 but not with HME17 (*c* in *C* and *D*). In the double staining of an atrial section using HME17 and HMC50 (*E, F*), myofibers reactive with HME17 were found to also be stained by HMC50 (*a* in *E* and *F*), but some myofibers also existed that were stained only by HMC50 but not by HME17 (*c* in *E* and *F*). Thus, in the atrium, it was suggested again that the binding of HMC14 and 50 to HCs was not disturbed by the presence of ventricular type LCI.

Similarly, since HAL8 stained almost all atrial myofibers diffusely (*G* in Fig. 9), but HMC14 and 50 did so heterogeneously (*E, F* in Fig. 2), the presence of ALCI appeared not to alter the binding of HMC14 and 50 to HCs in the atrium. Also, since HAL8 stained some ventricular myofibers heterogeneously (H) but HMC14 and 50 stained all ventricular myofibers homogeneously (*B, C* in Fig. 2), it was concluded again that the presence of atrial type LCI did not disturb the binding of MoAbs to HCs in the ventricle. Therefore, the redistribution of HC isoforms during the developmental stage and in re-

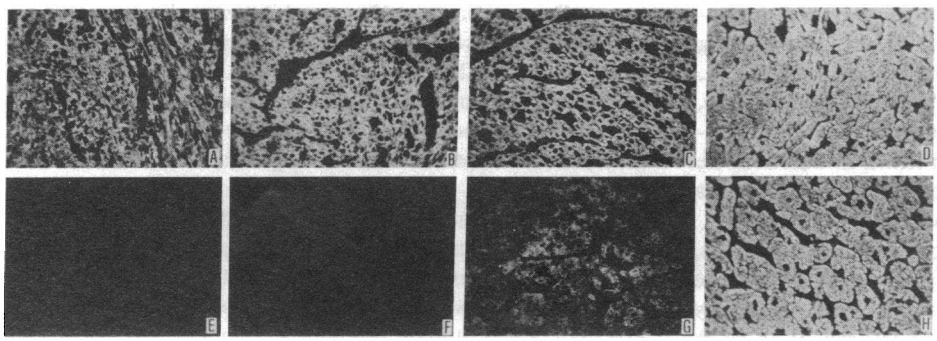

Figure 8. Fetal calf left ventricular myofibers stained by HMC14 (*A–D*) and HMC50 (*E–H*). (*A, E*) After 2 mo of gestation. *B, F:* 5 mo; *C, G:* 8 mo. *D, H:* adult. Although HMC14 stained ventricular myofibers of early embryos, HMC50 did not stain 2–5-mo ventricular myofibers, but began to stain 8-mo ventricular myofibers heterogeneously. In the adult ventricle, HMC50 stained all ventricular myofibers strongly as did HMC14.

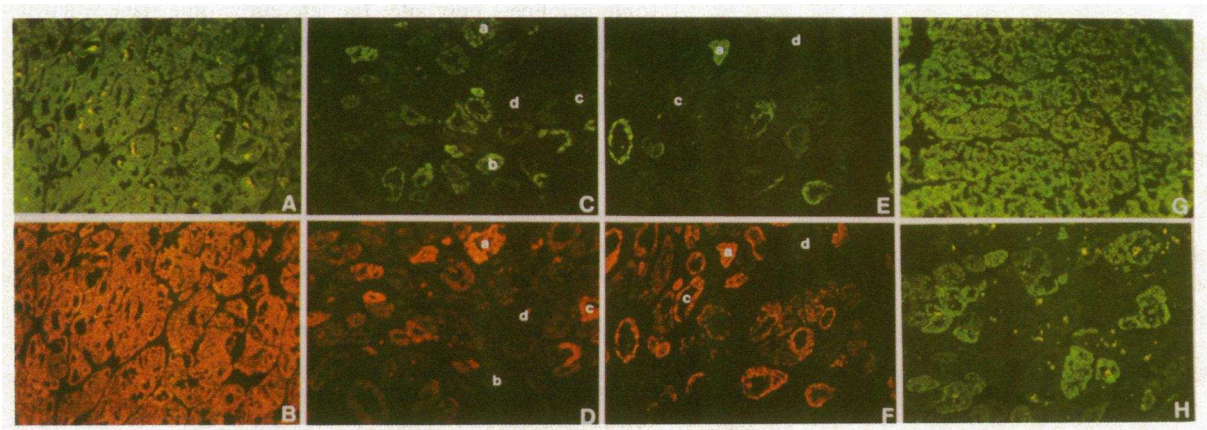

Figure 9. Distribution of atrial and ventricular type LCI isoforms in human hearts. (A, B) The same field of ventricular tissue double-stained by HME17 (*A*), specific for ventricular LCI, and HMC50 (B). HME17 and HMC50 were treated with FITC-labeled antimouse IgG_1 and TRITC-labeled antimouse IgG_{2b}, respectively. All myofibers were homogeneously reacted with both MoAbs. (*C, D*) The same field of atrial tissue double-stained by HME17 (*C*) and HMC14 (*D*). HME17 and HMC14 were treated with FITC-labeled antimouse IgG_1 and TRITC-labeled antimouse IgG_{2a}, respectively. Some myofibers (*a*) were stained by both MoAbs but another myofibers (*b, c*) reacted with only one of them. There existed myofibers (*d*) that did not react with both antibodies. (*E, F*) Same as in *C, D*, except that HMC50 and TRITC-labeled antimouse IgG_{2b} were used instead of HMC14 (*F*). Some myofibers (*a*) were stained with both MoAbs, but another (*c*) with only HMC50 without showing any reactivity with HME17. There exist some myofibers (*d*) that were not stained by both MoAbs. (*G, H*) Atrial (*G*) and ventricular tissue (*H*) stained by HAL8, specific for atrial LCI. Although HAL8 stained almost all atrial myofibers, it stained only some ventricular myofibers heterogeneously.

sponse to pressure overload were not caused by changes in LCI isoforms.

Discussion

The results presented here have clearly demonstrated the presence and the distribution of two subtypes of HCβ in the human heart, and their redistribution according to pressure overload and developmental stage. By isolation of each myosin through affinity columns, we also confirmed that these myosin HC molecules had differences in primary structure, suggesting that they were products of different mRNAs transcribed from different genes or from the same gene by alternative splicing. Recent reports have suggested the existence of heterogeneity of HCβ in bovine hearts by tryptic peptide mapping of myosin subfragment I (14). Determining the amino acid sequence of the carboxyl terminal 20,000-D tryptic heavy chain peptide from bovine ventricular myosin subfragment-1, Flink et al. found that at six positions in the sequence, two different amino acid residue were present, suggesting the presence of two different HCβ (14). Bouvagnet et al. also postulated the existence of two β-type HCs in the human heart in immunohistochemical studies (15). But, since the respective HCs have not been isolated yet, the possibility that the apparent heterogeneous staining might be posttranslational modifications of the same protein or alteration of antibody binding due to changes of some HC binding proteins was not ruled out. Using MoAbs specific for HCβ, we isolated, for the first time, two immunologically distinct HCβ molecules and demonstrated their distribution in the human heart. In addition, we were able to demonstrate that the expression of these HCβ was regulated by pressure-overload and developmental stage.

Although CMA19 and HMC50 clearly reacted with HCs, HMC14 did not react with the antigens on nitrocellulose membrane. We know that the specificity of HMC14 is very critical to confirm the existence of the heterogeneity of HCβ. An inhibition study by purified HC confirmed that the epitope detected by HMC14 was present in HC. Furthermore, the presence of a myofiber that reacted with HMC14 without reacting with CMA19 or HMC50 (Fig. 3) led us to believe that HMC14 was directed to HC. Finally, the fact that affinity purified myosin through a HMC14 column accompanied LCs indistinguishable from LCs of other types of myosin, but had HC peptide fragments different from the digests of other isomyosin, further confirmed that the epitope determined by HMC14 existed on the HC molecule. Thus, although HMC14 probably detected a tertiary structure, which is most likely to be affected by posttranslational modifications, the differences in proteolytic fragments displayed by three HC variants are most likely to be a consequence of differences in amino acid sequence and not a result of posttranslational modifications. But we could not confirm the existence of respective genes. To date, two HC genes encoding HCα and HCβ were isolated from small mammalian hearts, and they showed highly conserved sequence homology (6, 28). Therefore, it is not surprising that some peptide fragments produced by chymotriptic digestion were common to each other, as is demonstrated in Fig. 5. This is more likely if these proteins are not produced by different genes, but by alternative splicing of the same gene as is the case of the existence of three transcripts from the same gene in Drosophila (29). This possibility was also suggested by the presence of two HCα-mRNAs in S_1 mapping experiments in the rat heart (28, 30). Whether there exist more than two genes encoding cardiac HCs in the human heart, or whether there exists a mechanism of alternative splicing to produce different transcripts from the same gene should be resolved in the future.

The nomenclature of β_1 and β_2 was dependent on the immunohistochemical data in the present study. Their distribution in normal human hearts and redistribution in pressure-overloaded atria were characteristic to β-type myosin isozyme (10, 31). Since human ventricular myofiber was stained by

HMC14 and HMC50 diffusely and homogeneously, the possibility that one HCβ molecule in the ventricle contained epitopes detected by HMC14 as well as HMC50 is possible. However, a difference in the staining pattern during the developmental stage should eliminate this possibility. Therefore, it was reasonable to consider that two different HCβ molecules were present in ventricular myofibers. In this respect, our MoAbs are not identical to MoAbs used in the report of Bouvagnet et al., since their MoAbs stained ventricular myofibers heterogeneously (15). Although we don't know that our MoAbs corresponded to the two subtypes of HCβ reported by Flink et al. (14), our and their data support the existence of heterogeneity of HCβ in the ventricle.

In adult human hearts, HCα was expressed in almost all atrial myofibers and in a few ventricular myofibers. In contrast, β_1 and β_2 might coexist in all ventricular myofibers and also exist in some myofibers of the atrium, where, however, they were not necessarily expressed simultaneously in individual myofibers. Atrial myofibers containing β_2 appeared to be approximately two or three times as numerous as those expressing β_1. Although we could not quantify each isozyme accurately, we could determine the number of myofibers expressing each isozyme. Our data indicated that the number of myofibers expressing each isozyme were in order of: $\beta_1 = \beta_2 \gg$ HCα in the ventricle and HC$\alpha > \beta_2 > \beta_1$ in the atrium.

Recent studies have yielded evidence regarding isozymic redistribution induced by pressure-overload in laboratory animals (32, 33). In a previous report, we showed that a redistribution of isozymes from HCα to HCβ (β_1) can occur by pressure overload even in the human atrial myocardium (10). In this study, we examined whether β_2 showed a change similar to β_1 by pressure overload. In pressure-overloaded atria, the myofibers containing β_2 increased significantly, while those expressing HCα decreased, and the staining pattern with each MoAb resembled that observed in ventricles. The degree of the increment of β_2 correlated well with the mean atrial pressure when calculated as reactive myofibers or total scores. HCβ has a low ATPase activity that relates to the low velocity of shortening; hence it has an improved contraction for an equivalent amount of work in efficiency (34), and therefore, an isozymic redistribution from HCα to β_1 and β_2 is regarded as a compensative mechanism to adapt to increased pressure work. Thus, β_2 as well as β_1 might play a physiologically important role in pressure overload, and a balanced isozymic redistribution among HCα, β_1, and β_2 should contribute to adaptation to pressure overload in human atria. Although we know that HCα-containing myofibers decreased in pressure-overloaded ventricles obtained from patients with MS and/or MR, suggesting that an isozymic redistribution from HCα to HCβ also occurs in human ventricles (35), since HMC14 and 50 still stained myofibers of pressure-overloaded human ventricles homogeneously as in normal ventricles, we could not elucidate the change between β_1 and β_2. Further clarification by quantitative analysis of redistribution between β_1 and β_2 will require a new technique such as a radioimmunometric assay.

In rat and rabbit ventricles, HCβ, which is indistinguishable from HCβ expressed in adult hearts (36, 37), is known to be present in fetal hearts and to be replaced by HCα after birth (9, 36, 38). But in this study, we were able to demonstrate that HCβ expressed in fetal ventricles was clearly different from that expressed in adult ventricles. In fetal calf ventricles, β_2 did not exist or was present in very small quantities, whereas β_1 existed from the early embryonic stage. Therefore, although we could not find a fetal-specific myosin HC, a difference in the composition of subtypes of HCβ (β_1, β_2) between fetal and adult hearts was observed in this study. The physiological role of individual HCβ during development remained unknown; however, β_1 might be a physiological myosin isozyme in the special environment of fetal heart.

Finally, our results regarding isozymic redistribution according to pressure overload and developmental stage were derived from immunohistochemical data. Since myosin is composed of HCs and LCs, it is possible that alteration of LCs might alter the binding of each MoAb to HC and therefore give the impression of a change in HC isozymes. For example, the expression of LCI isoform has been reported to be switched by pressure overload and developmental stage, that is, ventricular type LCI replaces the atrial one in pressure-overloaded atria (27), and a fetal specific LCI indistinguishable from atrial LCI is expressed in embryonic ventricles (26). Therefore, the possibility mentioned above could not be ignored in the present study. However, MoAbs specific for either atrial or ventricular LCI (ALCI, VLCI), which were recently produced in our laboratory, resolved this problem. Since a MoAb specific for ALCI stained some ventricular myofibers heterogeneously and almost all atrial myofibers diffusely, it was suggested that ALCI or atrial type LCI did not alter the binding of each MoAb to HC. Similarly, a MoAb specific for VLCI stained all ventricular myofibers diffusely and some atrial myofibers heterogeneously, it was also suggested that VLCI or ventricular type LCI did not alter the binding of each MoAb to HC. Thus, alterations of LCI did not alter the binding of MoAb to HC.

In conclusion, in the human and bovine hearts, there are two immunologically distinct subtypes of HCβ. The expression of these isozymes may be regulated by at least two factors: developmental stage and pressure overload.

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